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# *In vitro* Evaluation of Inhibitory Potency of *Galium sinaicum* (Delile ex Decne) Boiss. Against Oxidation, Inflammation, and Enzymes Linked Diabetes Mellitus Type II

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## ABSTRACT

To study the control ability of *Galium sinaicum* on diabetes mellitus type II. *In vitro*, antioxidant, anti-inflammatory, α-amylase and α-glucosidase inhibitory activity of *Galium sinaicum* were evaluated. 80% hydroethanolic extract of *Galium sinaicum* (GsEE) possessed potent to moderate α-amylase and α-glucosidase inhibitory potency (78.95 and 60.02%) at 500µg/ml recording comparable $IC_{50}$ value of 57.84 ± 3.18µg/ml and 118.65± 1.04µg/ml, with acarbose, respectively. GsEE inhibited lysis of RBC induced by hypotonic solution, recording percentage of 81.93% comparing with aspirin (95.83%) at concentration 1000µg/ml with notable $IC_{50}$ value (48.05±0.48µg/ml) comparing with aspirin. GsEE highly succeeded comparing to gallic acid in neutralising DPPH radicals at concentration 300µg/ml till 700µg/ml, scavenging potency was ranged about 93%, recording $IC_{50}$ value of 120.6µg/ml comparing with gallic acid (2.8 µg/ml). Regarding to current study *Galium sinaicum* herb was proved to be antidiabetic controller, what support claims of being used as medicinal herbal tea for diabetics in Saint Catherine area in Saini, Egypt.

***Keywords:*** *Galium sinaicum,* antioxidants, anti-inflammatory, alfa-amylase, alfa-glucosidase

## 1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycaemia resulting from defects in insulin secretion or insulin action or both (American Diabetes Association, 2007). Diabetes mellitus has been categorized by World Health Organization, (2017) as one of the four major non-communicable diseases that should attract urgent attention from all key health foundations, where DM is announced as the third highest risk factor for worldwide premature mortality (Duncan, 2017). Data from the International Diabetes Federation (IDF) affirms that about 415 million people live with diabetes in the world with a prevalence rate of 8.8%. It is estimated that by 2040, about 642 million people will be diabetic with type 2 diabetes mellitus as the major type of diabetes (IDF, 2015). Factors such as genetic disposition, environmental factor, diet, physical inactivity and obesity do significantly contribute to the progression of insulin resistance and to the development of type II diabetes (Oguntibeju, 2019). Diabetes mellitus is described as a complex and multifactorial metabolic syndrome with characteristic abnormal metabolism in carbohydrates, fats and proteins leading to hyperglycaemia and hyperlipidaemia (Rehman and Akash, 2017). Later, scientific research proposed DM as an inflammatory condition (Navarro and Mora, 2005). Their argument is based on the hypothesis proposed by Crook who suggested that long-term innate immune system activation, resulting in chronic inflammation brings about a disease instead of repair, potentially resulting in the development of type II diabetes (Crook, 2004). Remarkably, research reports have shown that low-grade inflammation is associated with the risk of developing type II diabetes (Crook, 2004; Festa *et al.,* 2000; Frohlich *et al.,* 2000 and Hall, 2011). From another perspective, oxidative stress is confirmed as a known pathway in the pathogenesis of diabetic complications (Giacco and Brownlee, 2010). Where, prolonged exposure of human cells to hyperglycaemia leads to non-enzymatic glycation of proteins and the end products such as Schiff base and Amadori products, terminates in the production of reactive oxygen species (ROS) (Hojs *et al.,* 2016). Hyperglycaemic-induced oxidative stress is believed to increase the levels

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of pro-inflammatory proteins with infiltrated macrophages secreting inflammatory cytokines which leads to local and systemic inflammation (Wellen and Hotamisligil, 2005). Chronic inflammation and oxidative stress have been implicated in the pathopshysiology of diabetes mellitus. Complex interactions between oxidative stress and inflammatory pathways involve mechanisms for mutual amplification (positive feedback or a "vicious cycle"), triggering a series of complications such as coronary artery disease, cerebrovascular disease, renal failure, blindness, limb amputation, neurological complications, and pre-mature death (Lopez, 2001). Subsequently, arresting of elevated blood glucose in diabetic patients is vital to prevent these hyperglycaemic complications. The main therapeutic approach used to reduce blood sugar is stimulating insulin release, increasing the number of glucose transporters, inhibiting gluconeogenesis (Giacco and Brownlee, 2010). Additional therapeutic approach is via decreasing the postprandial glucose level in blood, which is attained by the gastrointestinal carbohydrate hydrolysing enzymes α-amylase and α-glucosidase enzyme inhibitors such as acarbose, voglibose and miglitol. Regardless of its effectiveness, the gastrointestinal side effects of synthetic drugs; including bloating, abdominal discomfort, diarrhoea, and flatulence make them a troublemaker for the patient (Chakrabarti and Rajagopalan, 2002). Nowadays, world is desperately looking for cure for serious diseases; scientists starts calling for mother nature for help. Legacy of medicinal plants along the time has secured treatment for many diseases among of them diabetes mellitus; hence, the ethnobotanical information reports about 800 plants possess antidiabetic potential (Paari and Pari, 2019)

*Galium sinaicum* (Delile ex Decne) Boiss is a wild herbal plant habituated in Saint Catherine protectorate, South Saini, Egypt. *G. sinaicum* belongs to Rubiaceae and like other *Galium sp*. characterized by presence of anthraquinones and lignens in root, and flavonoids plus iridoids in aerial part (El-Gamal *et al.,* 1999). Our study was established to assess α-glucosidase, α-amylase inhibition, antiinflammtory and antioxidant potentials of hydroethanolic extract of *Galium sinaicum* aerial part.

## 2. Materials and Methods

### 2.1. Plant Material

Aerial part of *Galium sinaicum* (Delile ex Decne) Boiss was collected in Spring from Wadi Gemal - Saint Katherine protectorate, South Saini- Egypt. Authentication of the plant was done by herbarium of Desert Research Center (DRC, Egypt). The plant was chopped into tiny bits, air-dried for two weeks and milled with a mechanical grinder. The ground plant (500g) was macerated in 80% ethanol for 24h., (3l×5). The extracts were filtered. The collected filtrate was concentrated under reduced pressure using a rotary evaporator (Buchi, R100, Switzerland) at an optimum temperature of 40–50°C and dried in a desiccator.

### 2.2. Chemicals

α-glucosidase (Saccharomyces cerevisiae), α-amylase (procaine pancreas), 3, 5, di-nitro salicylic acid (DNS), ρ-nitro-phenyl-α-D-glucopyranoside (p-NPG), and 1,1 diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. Sodium carbonate, sodium dihydrogen phosphate, and di-sodium hydrogen phosphate were purchased from Adwic, Egypt.

### 2.3. Amylase Inhibitor Activity

In a 96-well plate, reaction mixture containing 50µl phosphate buffer (100 mM, pH = 6.8), 10µl α–amylase (2U/ml), and 20µl of folded concentrations (1000-7.81µg\ml) of GsEE and acarbose as standard were preincubated at 37°C for 20min. A substrate, 1% soluble starch (20µl) (100 mM phosphate buffer, pH 6.8) was added. Reaction mixtures was incubated further at 37°C for 30min, 100µl of the 96mM 3,5-dinitrosalicylic acid (DNS) color reagent was added to mixtures and kept in a boiling water bath for 10min then left to cool (Telagari and Hullatti, 2015). Absorbance of the developed color was measured at 540nm using microplate reader (Biotek, ELx808IU, USA). Without test (extract and standard) substance was set up in parallel as control and each experiment was performed in triplicates. The results were expressed as percentage of inhibition, which was calculated using the formula, Inhibitory activity (%) = (1 − As/Ac) ×100 Where, As is the absorbance in the presence of test substance and Ac is the absorbance of control.

### 2.4. α-Glucosidase Inhibitory Activity

In a 96-well plate, reaction mixture containing 50 µl phosphate buffer (100 mM, pH = 6. 8), 10µl α-glucosidase (1U/ml), and 20µl of folded concentrations (1000-7.81µg\ml) of GsEE and acarbose as standard was preincubated at 37°C for 15min. Then, 20µl P-NPG (5 mM) was added as a substrate and incubated further at 37°C for 20min. The reaction was stopped by adding 50µl 0.1M $Na_2CO_3$ (Telagari and Hullatti, 2015). The absorbance of the released p-nitrophenol was monitored at 405nm using microplate reader (Biotek, ELx808IU, USA). Without test substance was set up in parallel as a control and each experiment was performed in triplicates. The results were expressed as percentage of inhibition, which was calculated using the formula, Inhibitory activity (%) = (1 − As/Ac) ×100 Where, As is the absorbance in the presence of test substance and Ac is the absorbance of control.

### 2.5. Membrane Stabilizing Activity

Blood (3ml) was freshly collected into heparinized tubes and centrifuged at 3000rpm for 10min. A volume of iso-saline equivalent to that of the supernatant was used to dissolve the red blood pellets. The volume of the dissolved red blood pellets obtained was measured and reconstituted as 10% v/v suspension with isotonic buffer solution (10mM sodium phosphate buffer, pH7. 4). The buffer solution was constituted of 0.2g of $NaH_2PO_4$, 1.15g of $Na_2HPO_4$ and 9g of NaCl in 1l of dist. water. The reconstituted red blood cells were used as such. test sample consisted of 10% v/v RBCs suspension (0.5ml) mixed with 5ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing GsEE or aspirin (1000 - 7.8µg/ml). The control sample consisted of 0.5ml of 10% v/v RBCs suspension mixed with hypotonic-buffered saline solution alone. The mixtures were incubated for 10min at room temperature and centrifuged for 10min at 3000rpm and the absorbance of the supernatant was measured at 540nm using Unicam UV/Vis spectrophotometer (Umukoro and Ashorobi, 2006). The percentage of haemolysis inhibition or membrane stabilization was calculated by the following equation: % haemolysis Inhibition = 100 x ($OD_1$-$OD_2$/$OD_1$) Where: $OD_1$ = optical density of hypotonic-buffered saline solution alone, $OD_2$ = optical density of test sample in hypotonic solution. $IC_{50}$ value was calculated by sigmoidal non-linear regression model utilizing GraphPad (Version 4.0; San Diego, USA, www.graphpad.com).

### 2.6. Free Radical Scavenging Activity

A volume of 2.5ml of different concentrations of GsEE and Gallic acid (10-700µg/ml, in ethanol) were added to ethanolic solution of DPPH (0.3mM, 1ml). After 30min at room temperature in a dark cabinet, the absorbance values were measured at 517nm on a Unicam UV/Vis spectrophotometer. Ethanol (1ml) plus plant extract solution (2.5ml) was used as a blank, while DPPH solution plus methanol was used as a control (Katalinic *et al.,* 2006). Percentage of radical scavenging activity was calculated from the following equation: DPPH radical scavenging capacity (%) = [1-($Ab_{sample}$ – $Ab_{blank}$) / $Ab_{control}$]×100. $IC_{50}$ value was calculated by sigmoidal non-linear regression model utilizing GraphPad (Version 4.0; San Diego, USA, www.graphpad.com)

## 3. Results and Discussion

### 3.1. *In Vitro* α-Amylase Inhibition

Salivary and pancreatic $\alpha$-amylase catalyses the hydrolysis of $\alpha$-1, 4-glucosidic linkages of polysaccharide such as starch and glycogen. Subsequently, the $\alpha$-glucosidase hydrolyses the resulting oligosaccharides into glucose. $\alpha$-amylase inhibitory property of GsEE is presented in fig (1). The result revealed that the tested extract concentration dependently inhibited $\alpha$-amylase activity *in vitro*. At 500 µg/ml, GsEE proved challenged inhibition potency (78.95%) when comparing with acarbose at the same concentration (76.14%). GsEE exhibited a comparable $IC_{50}$ value of 57.84 ± 3.18µg/ml with the standard positive control acarbose that showed an $IC_{50}$ value of 40.19 ± 1.63 µg/ml (table 1).

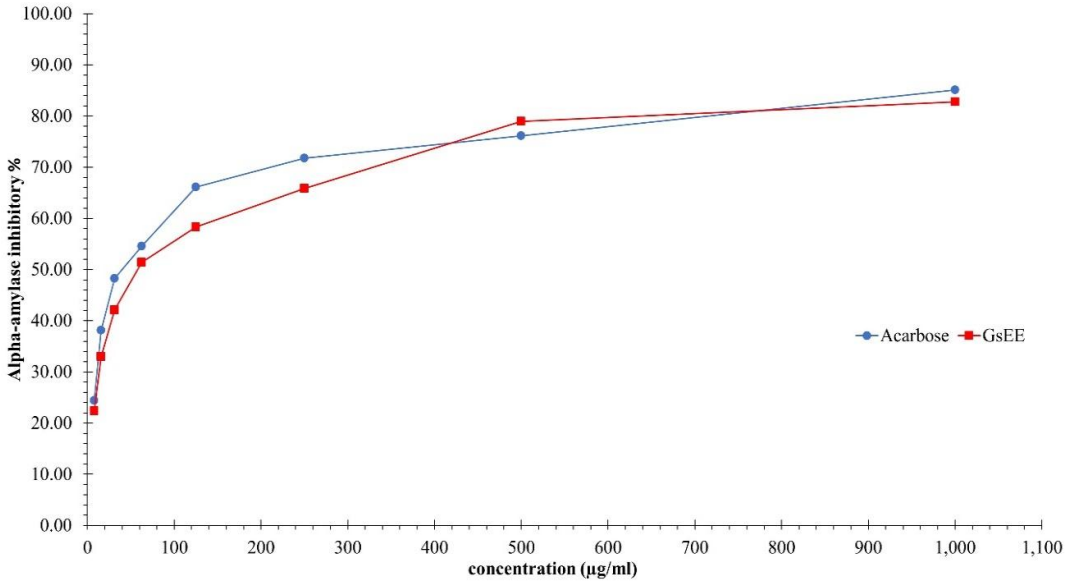


**Fig. 1:** α-amylase inhbition activity by GsEE. acarbose is used as standered inhbitor; data are presented as percentage of α-amylase inhibition (n=3)

**Table 1:** $IC_{50}$ (µg/ml) of α amylase, α glucosidase inhibitory, membrane stabilizing, and radical scavenging activity of GsEE

| | **α-amylase inhibitory activity $IC_{50}$** | **α-glucosidase inhibitory activity $IC_{50}$** | **Membrane stabilizing activity $IC_{50}$** | **DPPH scavenging activity $IC_{50}$** |
|---|---|---|---|---|
| **GsEE** | 57.84±3.18 | 118.65±1.04 | 48.05±0.48 | 120.6±1.20 |
| **Acarbose** | 40.19±1.63 | 29.34±1.11 | --- | --- |
| **Aspirin** | --- | --- | 17.49±0.37 | --- |
| **Gallic acid** | --- | --- | --- | 22.8±0.92 |

### 3.2. *In Vitro* α-Glucosidase Inhibition

α-glucosidase is a key enzyme of carbohydrate digestion which is in the brush-border surface membrane of intestinal cells. α-Glucosidase inhibitors block the actions of α-glucosidase enzymes in the small intestine, which limits the conversion of oligosaccharides and disaccharides to monosaccharides, to be absorbed. Agents with α-glucosidase inhibitory activity are useful as oral hypoglycaemic agents for governing postprandial hyperglycaemia (PPHG) in T2DM patients. Many plants have been reported with α-glucosidase inhibitory activity (Sunil *et al.,* 2019). Likewise, GsEE showed respectable dose dependent α-glucosidase inhibitory activity as shown in Fig (2), where inhibition percentage was in proportional to concentration. GsEE recorded moderate inhibition percentage (60.02%) at 500µg/ml compared with acarbose (80 %) at the same concentration. The $IC_{50}$ values were calculated (table 1). GsEE proved a reasonable $IC_{50}$ value of 118.65± 1.04µg/ml when compared with acarbose that displayed $IC_{50}$ value of 29.34±1.11µg/ml. inhibitors of pancreatic and hepatic glucosidase may be effective in retarding glucose absorption to suppress PPHG. As in T2DM, excessive hepatic glycogenolysis and gluconeogenesis in liver is associated with decreased utilization of glucose by tissues being the central mechanism expressing hyperglycaemia. Inhibition of these enzyme systems decreases the current blood glucose levels in diabetic patient (as a short-term effect) and shows a small reduction in haemoglobin A1c level (as a long-term effect) (Petersen *et al.,* 2017).

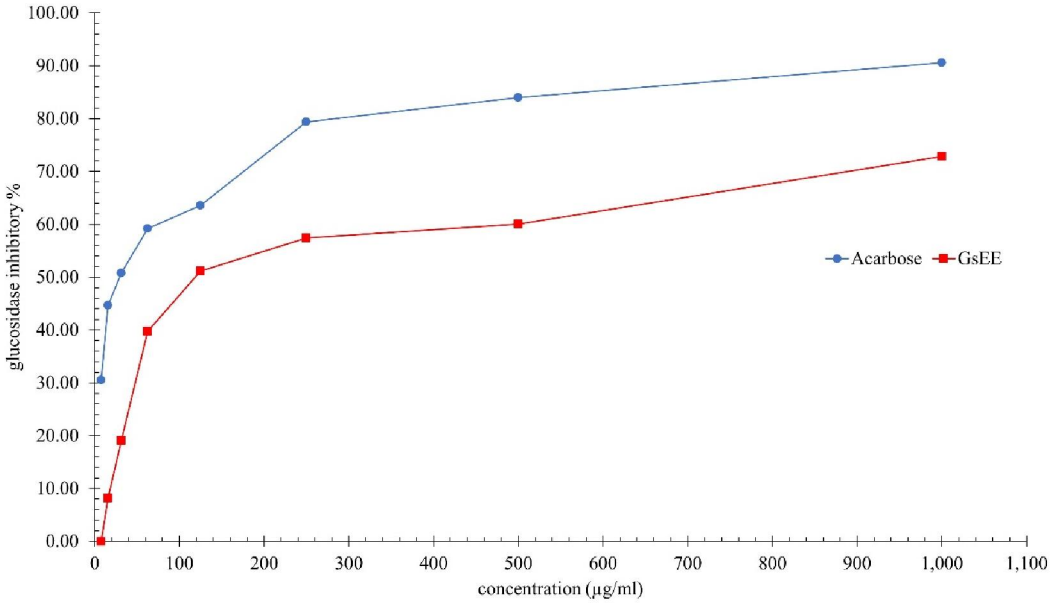


**Fig. 2:** α-glucosidase inhbition activity by GsEE. acarbose is used as standered inhbitor; data are presented as percentage of α-glucosidase inhibition (n=3)

### 3.3. Membrane Stabilizing Activity

Inflammation has been implicated as an important aetiological factor in the development of both insulin resistance and type II diabetes mellitus. This conclusion is largely deducted from studies demonstrating associations between elevated levels of circulating acute phase inflammatory markers and the development of T2DM (Greenfield and Campbell, 2006). A study by Pradhan *et al.,* (2001) showed that increased inflammatory biomarkers predicted insulin resistance and the development of T2DM. There is also a study that reported on the correlation between fasting insulin levels and C-reactive protein levels in plasma of diabetics showing that insulin resistance and inflammatory processes are linked.

Membrane stabilization assay of erythrocytes is a very current tool to investigate the anti-inflammatory potential of the plant extract *in vitro*. Exposure of red blood cells (RBCs) to stressful condition such as hypotonic medium, heat, methyl salicylate or phenyl hydrazine leads to lysis of the membranes accompanied by leakage of serum protein and fluids into the tissues starting inflammation (Halliwell and Whiteman, 2004). Data postulated in fig (3) showed that GsEE significantly ($p \leq 0.05$) inhibited lysis of RBC induced by hypotonic solution. This is shown by the high inhibition percentage of haemolysis that reached to 81.93% comparing with aspirin (95.83%) at concentration 1000µg/ml. the inhibition of lysis and haemolysis was presented as dose dependent behaviour in a range of folded concentrations (7.81-1000µg/ml). GsEE presented a notable $IC_{50}$ value of 48.05±0.48µg/ml comparing with aspirin that recorded 17.49±0.37µg/ml (table 1). It is well known that injury to red cell membrane will put the cell in a ready mode to secondary damage through free radicals induced lipid peroxidation (Halliwell and Whiteman, 2004). Membrane stabilizing agents halt leakage of serum protein and fluids into the tissues during the period of increased permeability caused by inflammatory mediators (Chaitanya *et al.,* 2011). GsEE possibly stabilized RBC membrane by preventing the release of lysosomal enzymes and active mediators of inflammation.

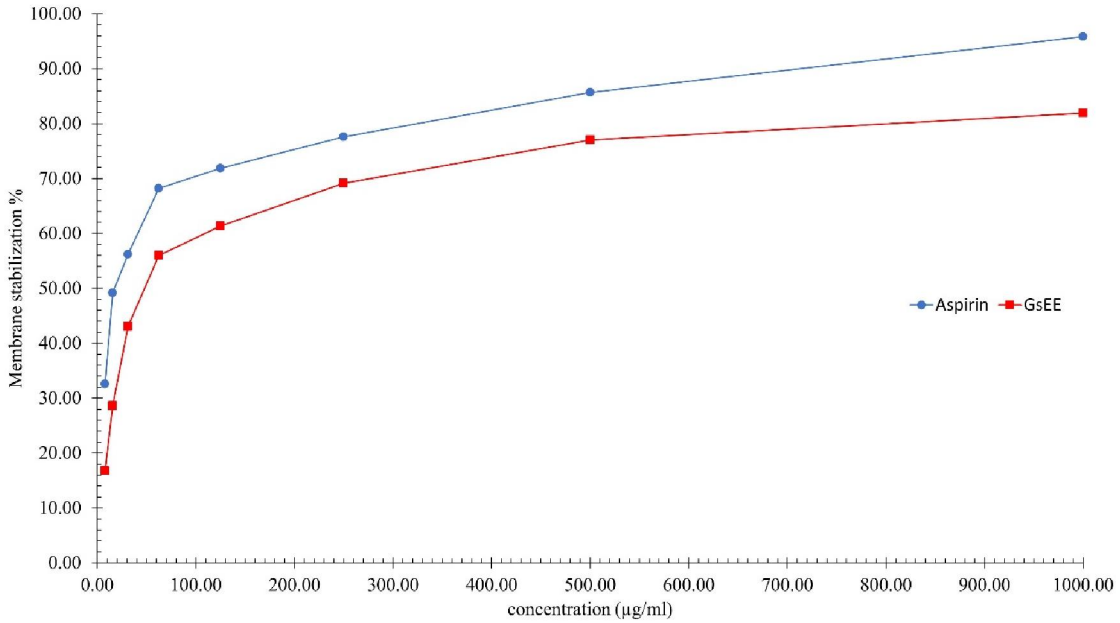


**Fig. 3:** Membrane stabilizing activity by GsEE. aspirin is used as standared; data are presented as percentage of membrane stabilization (n=3)

### 3.4. Radical Scavenging Activity

Free radicals are molecules with one or more single pair of the electron that can rapidly react with the constituents such as proteins, nucleic acid, and lipids. These reactive particles are generated in cellular membrane, mitochondria, nucleus, lysosome, peroxisome, endoplasmic reticulum, and cytoplasm. The enhanced generation of the reactive species implicated with hyperglycaemia (Halliwell and Gutteridge, 1999).

Former studies proved that overproduction of free radicals and defeat of antioxidants protection system are contributed in pathogenesis of diabetes (Opara, 2002). The mechanism behind the prooxidant-antioxidant imbalance in diabetes mellitus is auto-oxidation of glucose, increased the formation of advanced glycation end products (AGEs), polyol pathway, hexosamine pathway, and mitochondrial respiratory chain (Singh *et al.,* 2009).

Therefore, regular intake of antioxidants along with anti-diabetic drugs are frequently recommended to avoid such complications. GsEE highly succeeded comparing to gallic acid in neutralising DPPH radicals, dramatically bleached purple colour in concentration dependant manner as shown in fig (4). Plateau pattern recognised at concentration 300µg/ml till 700µg/ml with scavenging potency ranged about 93%, recording $IC_{50}$ value of 120.6µg/ml and 22.8µg/ml for gallic acid as control (table 1). It is worth mentioning, that antioxidants therapy defends the beta-cell against oxidative stress-induced apoptosis and preserves the function of the beta-cell. Moreover, data from earlier studies showed the antioxidants diminish diabetic-allied serious complications and recover insulin sensitivity (Rajendiran *et al.,* 2018). Based on current data, *G. sinaicum* in this study is presented as antidiabetic, anti-inflammatory, and antioxidant herb that may attributed to phenolics content. Earlier studies have strongly suggested a connection between polyphenolic content of the extract and inhibition of α-amylase and α-glucosidase activity (Yoshikawa *et al.,* 2001; McDougall *et al.,* 2005 and Mai *et al.,* 2007), furthermore, Ramkumar *et al.,* established a positive and significant correlation between polyphenolic content and α-glucosidase inhibition ($r^2 = 0.92$) as well as α-amylase inhibition ($r^2 = 0.97$) (Ramkumar *et al.,* 2001). It has been suggested that the mechanism of inhibition of *α*-amylase may occur through the direct blockage of the active centre at several subsites of the enzyme as also suggested for other inhibitors (McCue and Shetty, 2004).

From another side, many flavonoids and other phenolics have been proved their remarkable curative effect on inflammatory processes like quercetin, apigenin, hesperidin and luteolin (Kumar and Pandey, 2013 and Locatelli, *et al.,* 2018). Also, earlier research emphasized on plants with high levels of

phenolic compounds exhibit high antioxidant activity (Kumar *et al.,* 2015 and Sunil *et al.,* 2019). Accordingly and based on HPLC fingerprint of hydroethanolic extract of *G. sinaicum,* it was found that phenolic pool enclosed gallic acid, chlorogenic acid, catechin, caffeic acid, syringic acid, coumaric acid, vanillin, ferulic acid, propyl gallate, cinnamic acid, rutin, naringenin, 4`.7-dihydroxyisoflavone, and quercetin (Sobieh and Fahmy, 2020), besides, *G. sinaicum* was proved to have 358.33 ± 1.2mgeq. gallic/g d.w phenolics also *G. sinaicum* was proved as a mediator of blood sugar level in vivo (Elhaw and Aldinary, 2018).

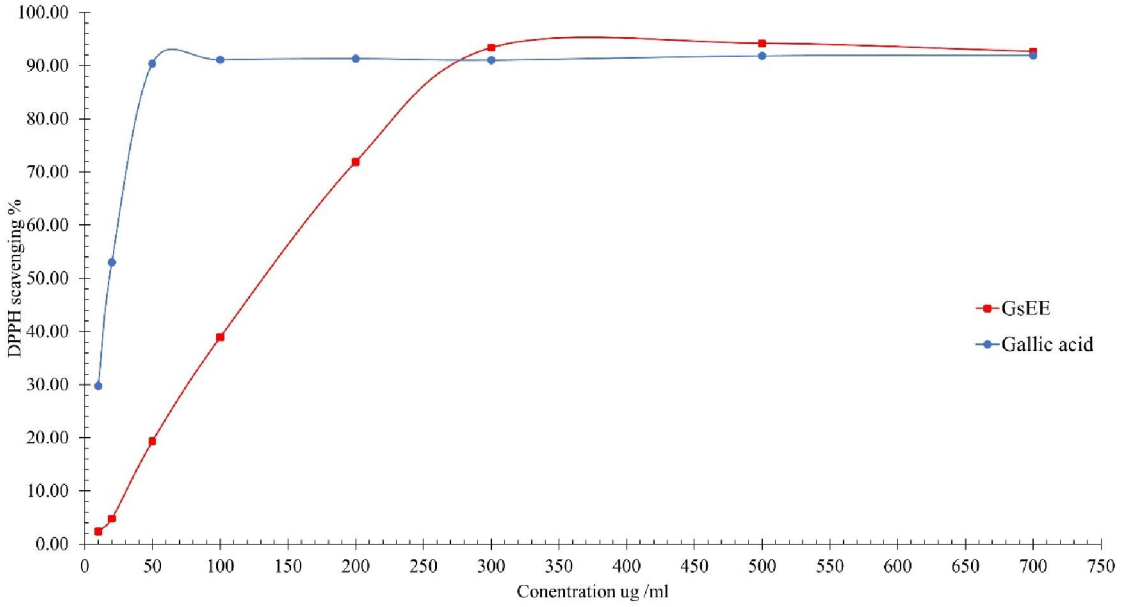


**Fig. 4:** DPPH radical scavenging activity of GsEE. gallic acid is used as standared scavenger; Data are presented as the percentage of DPPH radical scavenging (n=3)

## 4. Conclusion

Traditional medical practitioners claim that *G. sinaicum* herbal tea is used to sustain hyperglycaemia in diabetic patients in saint Catherine area To correlate, in this study, *G. sinaicum* is proved to be promising as a medicinal plant, simply may has the ability to stop α-amylase, and α-glucosidase actions, arresting postprandial hyperglycaemia, control inflammation and oxidative stress which are crucial for T2DM complications development, that all may be attributed to richness of phenolic pool in *G. sinaicum.*

## 5. Acknowledgment

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