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Evaluation of antioxidant and Antidiabetic properties of goa 111, a mixture of *Gymnema sylvestre*, *Ocimum sanctum* and *Azadirachta indica* extract (1:1:1): An *in vitro* approach

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Abstract

Type 2 diabetes mellitus once considered as a metabolic disorder characterized by chronic elevation in both fasting and postprandial levels is now established as a polygenic disease involving several etiological factors. Alpha amylase and alpha glucosidase inhibitors are used to achieve greater control over hyperglycemia in type 2 diabetes mellitus. Polyherbal preparations with various active principles and properties have been used in different regions and cultures to treat a wide range of human diseases. In the present study an attempt has been made to evaluate the antioxidant and *in vitro* antidiabetic efficacy of GOA 111, a mixture of *Gymnema sylvestre*, *Ocimum sanctum* and *Azadirachta indica* in the ratio of 1:1:1. Polyherbal formulation was analyzed for phytochemicals. The antioxidant potential of GOA 111 was determined using DPPH, ABTS assay. The effect of GOA 111 on the activities of alpha glucosidase and alpha amylase were studied. GOA 111 contains Alkaloids, flavonoids, tannins, saponins, glycosides, steroids, phenols, resins, Coumarins, proteins and carbohydrates. Its shows significant amounts of total phenol, flavonol and flavonoid. There was a significant percentage inhibition of 74% and 72% at a concentrations of 1000µg/ml for DPPH and ABTS assays respectively. GOA 111 showed 58 % inhibition and 72% inhibition for alpha amylase and glucosidase assays at a concentration of 500 µg/ml. The formulation, in different concentrations, is able to enhance uptake of glucose in yeast, the simplest eukaryotic cells. Hence it can be concluded that Goa 111 possess significant antidiabetic and antioxidant properties.

Keywords: Poly herbal formulation, GOA 111, alpha amylase, alpha glucosidase, antioxidant activity the

Introduction

Nature is a vital system, which shapes the backdrop for the emergence, evolution and existence of life and it is a good source for a wide array of effective medicines without side effects. The medicinal plants used in for the treatment of various non-communicable diseases including diabetes and cancer are known to exert their pharmacological properties through the synergistic action of various Phyto ingredients present in them. Polyherbal preparations having various active ingredients with biological properties have been used in different regions and cultures to treat a wide range of human diseases [1]. Polyherbal preparations are the collection of therapeutic entities that are formulated and blended properly on the basis of healing properties of individual phytochemicals with respect to the pathological conditions prevailing at the time of diagnosis. Accordingly, the polyherbal components used in the present study was formulated based on the earlier reports on the pharmacological properties of *Gymnema sylvestre*, *Ocimum sanctum* and *Azadirachta indica* in the ratio of 1:1:1.

Polyherbal preparations are the collection of therapeutic entities that are formulated and blended properly on the basis of healing properties of individual phytochemicals with respect to the pathological conditions prevailing at the time of diagnosis. Accordingly, the polyherbal components used in the present study was formulated based on the earlier reports on the pharmacological properties of *Gymnema sylvestre*, *Ocimum sanctum* and *Azadirachta indica* in the ratio of 1:1:1.

G. sylvestre (Asclepiadaceae) a vulnerable species is a slow growing, perennial, medicinal woody climber found in central and peninsular India. Its leaves, called "Gurmar" in India, are well known for their sweet taste suppressing activity [2] and are used for the treatment of Diabetes mellitus [3-4] for over 2000 year, hence the name "Gurmar" meaning 'sugar destroying'. It is used in food additives against obesity. *G. sylvestre* is a woody, climbing herb indigenous to the tropical forests of central and southern India. *G. sylvestre* is one of the Indispensable medicinal plants used in Ayurvedic system of medicine for the treatment of diverse diseases. It is used in food additives against obesity [5-6].

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Ocimum sanctum belongs to the family of Lamiaceae and is commonly known as Thulasi/Tulsi in India. It is widely grown in all regions and is considered as the sacred plant of India. Tulsi or Holy Basil is a sacred plant used by Vaishnavas for thousands of years. In the Indian sub-continent, fresh leaves of this plant are most commonly used for the treatment of cough, cold, abdominal pain, skin diseases, arthritis, painful eye diseases, measles, and diarrhea. The preclinical evaluation on various extracts of different parts of *Ocimum sanctum* showed antioxidant, anti-cancer, anti-diabetic, anti-bacterial, hepatoprotective and cardio protective actions [7-11].

Azadirachta indica, commonly known as neem, has attracted worldwide prominence in recent years, owing to its wide range of medicinal properties. Neem has been extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a cynosure of modern medicine [12] (Subapriya and Nagini, 2005). More than 140 compounds including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids, ketones azadirachtin and nimbin have been isolated from different parts of neem [13]. Almost every part of this tree is used in different diseases as traditional drug. The tree and its extracts have also been reported to possess insecticidal, anti-viral, anti-fungal and anti-bacterial properties [14] having known the pharmacological and beneficial properties of *G. sylvestrea*, *O. sanctum* and *A. indica*, the present study was aimed to evaluate the *in vitro* antioxidant and antidiabetic properties of GOA 111 systematically.

Materials and methods plant material

A mixture of *G. sylvestrea*, *O. sanctum* and *A. indica* in the ratio of 1:1:1, was used in the present study

Preparation of aqueous herbal extract

10g of GOA 111 powder was immersed in 100 ml of distilled water and left in a orbital shaker for 2 hours (100mg/ml) was used for all further analysis.

Phytochemical screening

Polyherbal formulation (GOA 111) was subjected to qualitative phytochemicals screening for the presence of major phytochemicals [15-16].

Determination of total phenol

The total phenolic content in different solvent extracts was determined with the Folin- Ciocalteu's reagent (FCR). In the procedure, different concentrations of the extracts were mixed with 0.4ml FCR (diluted 1:10v/v) was added. 4ml of sodium carbonate solution was added after 5 min and the final volume was made up to 10ml with distilled water and allowed to stand for 90 minutes at room temperature. Absorbance of sample was measured against the blank at 670 nm and total phenolic content of the extract was expressed in terms of milligrams of gallic acid per gram of dry weight [17].

Total flavonoid and Flavonols determination

Total flavonoid content was determined by Aluminium chloride method using quercetin as a standard. To 1ml of test sample, 4ml of water were added to a volumetric flask and 0.3 ml of 5% sodium nitrite was added. 0.3ml of 10% Aluminium chloride was added after 5 min and incubated at room temperature for 6 min, 2ml of 1M Sodium hydroxide was added to the reaction mixture and the final volume was made up to 10ml with distilled water. The absorbance of the

reaction mixture was measured at 510nm against a blank colorimetrically. Results were expressed as quercetin equivalents (mg quercetin/g dried extract).

Total flavonols were estimated by mixing test solution with 2ml (20 gm/l) AlCl₃ and 6 ml (50 gm/l) sodium acetate. The absorption at 440nm was read after 2.5h at 20°C expressed as mg of quercetin per gram of dry extract by the method of Miliuskas *et al.* 2004 [18]

Assessment of *in vitro* antioxidant activity

Evaluation of antioxidant activity by DPPH and ABTS radical scavenging method

The DPPH radical scavenging activity of extract was determined by the method of Brand-Williams *et al.*, 1995 with some modifications [19]. ABTS radical scavenging activity of extract was determined according to the method of Re *et al.*, 1999 [20].

Evaluation of *in vitro* anti-diabetic potential

Glucose uptake by yeast cells

Yeast suspension was prepared by repeated washing at 3000g for 5 min in distilled water until the supernatant fluids were clear. A 10% (v/v) suspension was prepared with the supernatant fluid. 1mL of the glucose solutions (10 and 25 mM) was added to various concentrations of the extract (250, 500, 750, and 1000 µg) and incubated at 37°C. Reaction was started by adding 100 µl of yeast suspension, vortexed and further incubated at 37°C for 60 min, and centrifuged at 2,500g for 5min and the glucose content was estimated in the supernatant. The percentage increase in glucose uptake by yeast cells was calculated using the following formula;

$$\% \text{ inhibition} = \frac{\text{absorbance sample} - \text{absorbance control}}{\text{Absorbance sample}} \times 100$$

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 [21].

Inhibition of alpha amylase

100 µl of test samples and standard drug (100-500µg/ml) were taken. Then 250 µl of α- amylase (1 mg/ml) in 0.2 M sodium phosphate buffer (pH 6.9) was added to each tube and were incubated at 37°C for 20 min. Then 250µl of a 0.5% starch solution in 0.2 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 37°C for 15 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid. The test tubes were then incubated in a boiling water bath at 100°C for 10 min, cooled to room temperature. The reaction mixture was then diluted to 10 ml using distilled water and absorbance was measured at 540 nm [22].

$$\% \text{ of alpha amylase inhibition} = \frac{\text{OD test} - \text{OD control}}{\text{OD test}} \times 100$$

Inhibition of alpha glucosidase

50 µl of 0.2 M sodium phosphate buffer (pH 6.8) and 50 µl of 0.1 U glucosidase was taken in different tubes. To this 50 µl of sample and standard of different concentrations were added (should not mix) and incubated at 37°C for 5 min. Then 50 µl of p-nitrophenyl alpha- D- glucosidase was added, vortexed and incubated at 37°C for 30 min. 50 µl of 0.1 M sodium carbonate was added. Absorbance was measured at 405 nm [23].

$$\% \text{ of alpha glucosidase inhibition} = \frac{\text{OD control} - \text{OD test}}{\text{OD control}} \times 100$$

Results and Discussion

Phytochemical analysis is of paramount importance in identifying new source of therapeutically and industrially

valuable components in traditional medicinal plants. In the present investigation, the primary and secondary metabolites were qualitatively and quantitatively analyzed in a polyherbal formulation.

Table 1: Phytochemical Screening of Herbal Powder Extract

S. No	Phytochemicals	Name of test	Inference
1	Alkaloids	Wagner's test	+
2	Flavonoids	1% ammonia	+
3	Glycosides	Legal's test	+
4	Saponins	1% lead acetate	+
5	Tannins	5% FeCl ₃	+
6	Cardiac glycosides	Keller killain test	-
7	Steroids	acetic anhydride	+
8	Phenol	ferric chloride and ferri cyanide	+
9	Anthraquinones	benzene	Trace
10	Resin	copper sulphate	+
11	Carboxylic acid	sodium bicarbonate	+
12	Coumarin	Alc. potassium hydroxide	+
13	Quinones	Alc. potassium hydroxide	-
14	Antho cyanine	HCl and ammonia	-
15	Diterpenes	copper acetate	-
16	Protein	Conc. nitric acid	+
17	Carbohydrate	Benedict's test	+

The results presented (table 1) reveal the presence of maximum number of plant constituents such as Alkaloids, flavonoids, tannins, saponins, glycosides, steroids, phenols, resins, Coumarins, proteins and carbohydrates.

Phytochemicals are ecologically derived non-nutrient secondary metabolites in plants which provide much of the colour and taste in fresh or processed food, fruits and

vegetables (Hussain *et al.*, 2013). Most of the phytochemicals such as polyphenols, glycosides, alkaloids, terpenoids, flavonoids, polysaccharides, tannins and saponins have been proposed for the treatment of various human ailments due to their significant antioxidant and related pharmacological properties [24, 25].

Table 2: The amount of total phenolics, flavonoid and Flavonols content is presented

Phytochemical	Quantity
Total phenol	50 ± 1.2 mg of gallic acid equivalent/gm of dry material
Total flavonoids	49.02 ± 0.78 mg of quercetin equivalents/gm of dry material
Flavonols	62.5 ± 0.41 mg of quercetin equivalents/gm of dry material

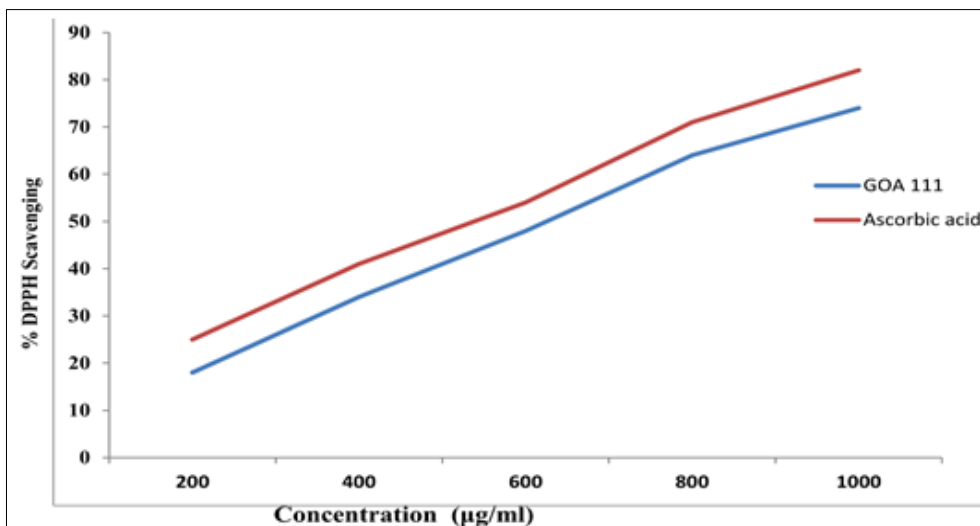
Table 3: depicts the quantitative estimation of enzymatic and non-enzymatic antioxidants

Assay of superoxide dismutase	Assay of catalase	Estimation of vitamin C
50% inhibition at a concentration of 0.5 mg of extract equivalent to 3 units of SOD	3.83 micromoles of H ₂ O ₂ / min	10.4 mg of Ascorbic acid / gm of the extract

Antioxidant activity of Goa 111

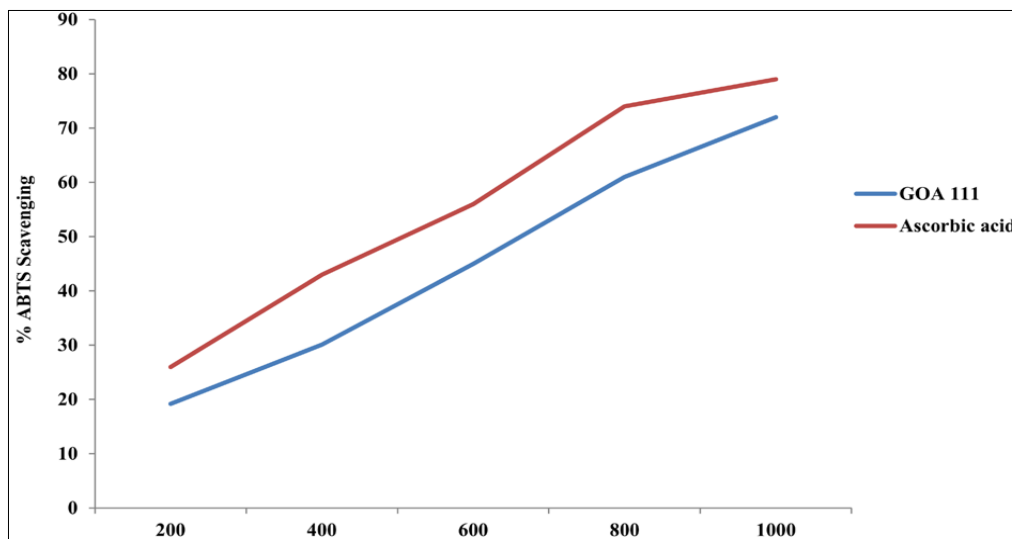
Figure 1 and 2 shows the dose dependent effect of *GOA 111* on the percentage inhibition of DPPH and ABTS radicals

present in the reaction mixtures, respectively. *GOA 111* scavenges DPPH and ABTS radical in a concentration dependent manner.



- The percentage inhibition at a concentration of 200ug /ml (18%) 400ug /ml (34%) 600ug /ml (48%) 800ug /ml (64%) 1000ug /ml (74%) were observed
- The Ic 50 value was found to be 633.80ug /ml

Fig 1: Dpph Radical Scavenging Potential of Goa 111



- The percentage inhibition at a concentration of 200ug /ml (19.2%) 400ug /ml (30.1%) 600ug /ml (45%) 800ug /ml (61%) 1000ug /ml (72%) were observed.
- The Ic 50 value was found to be 633.80ug /ml.

Fig 2: ABTS radical scavenging potential of Goa 111

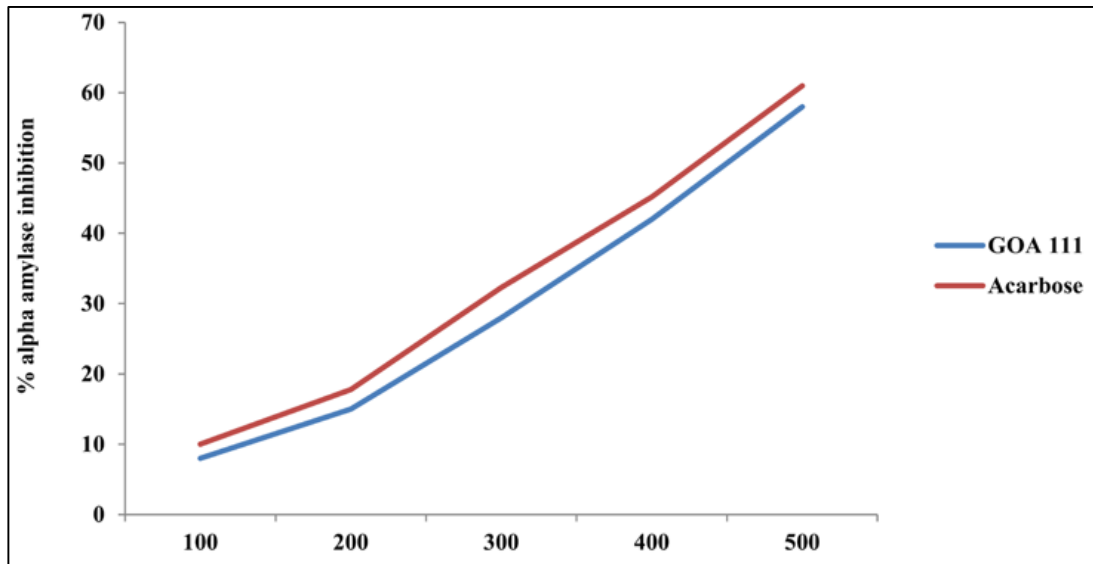
DPPH radical exhibits deep violet color in solution and become colorless or pale yellow when neutralized and converted into DPPH-H. Plants have been widely reported for their DPPH radical scavenging properties *in vitro* [26, 27]. ABTS radicals involve a single electron transfer process which is pre-formed radical monocation of ABTS radical which is generated by oxidation of ABTS radicals with potassium per sulfate and is reduced in the presence of such hydrogen-donating antioxidants. The antioxidant activity of different lead molecules depends on the number and location of hydroxyl and other functional groups such as carboxyl and phenolic ring system [28].

In DPPH assay, The percentage inhibition at a concentrations of 200µg/ml (18%) 400µg/ml (34%) 600µg/ml (48%) 800µg/ml (64%) 1000µg/ml (74%) were observed. The IC50 value was found to be 633.80 µg/ml. In ABTS assay, the percentage inhibition at a concentrations of 200µg/ml (19.2%) 400µg/ml (30.1%) 600µg/ml (45%) 800µg/ml (61%) 1000µg/ml (72%) were observed. The IC50 value was found

to be 633.80 µg/ml. Hence it is observed that GOA 111 possess significant free radical scavenging potential which might be due to the high content of phenolic and flavonoid in them.

Evaluation of anti-diabetic potential of Goa 111

Percent alpha amylase inhibition of the GOA 111 was plotted as a function of concentration in comparison with Acarbose as Figure 3. The results indicate that GOA efficiently inhibited alpha amylase enzyme *in vitro*. There was a dose dependent increase in percentage inhibitory activity against alpha amylase. The percent inhibition of GOA 111 was 8 % (100 µg) 15% (200 µg), 28 % (300 µg), 42 % (400 µg) and 58% (500 µg/ml). GOA 111 showed an IC50 value of 283.08µg/ml in the alpha amylase inhibition assay. The percent inhibition of Acarbose was 10% (100 µg) 17.8 % (200 µg), 32.3 % (300 µg), 45.2% (400 µg) and 61% (500 µg/ml). Acarbose showed an IC50 value of 430.06 µg/ml in the alpha amylase inhibition assay.

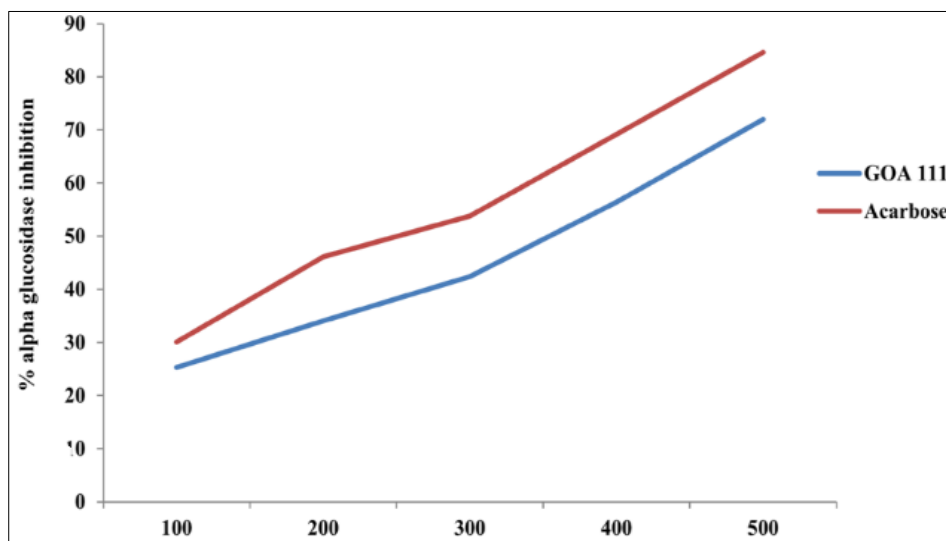


- The percent inhibition of GOA III was 8 % (100 pg) 15% (200 pg), 28 % (300 pg), 42 % (400 pg) and 58 (500 pg/ml).
- GOA III showed an IC₅₀ value of 283.08pg/ml in the alpha glucosidase inhibition assay.
- The percent inhibition of Acarbose was 10% (100 pg) 17.8 % (200 pg), 32.3 % (300 pg), 45.2% (400 pg) and 61% (500 pg/ml). A
- Acarbose showed an IC₅₀ value of 430.06 pg/ml in the alpha glucosidase inhibition assay.

Fig 3: α amylase inhibitory activity of GOA 111

Percent alpha glucosidase of the GOA 111 is shown in Figure 4. The percent inhibition of GOA 111 was 25.3% (100 μ g) 34.07% (200 μ g), 42.4% (300 μ g), 56.4% (400 μ g) and 72 (500 μ g/ml). GOA 111 showed an IC₅₀ value of 533.04 μ g/ml in the alpha glucosidase inhibition assay. The percent

inhibition of Acarbose was 10% (100 μ g) 46.1 % (200 μ g), 53.8% (300 μ g), 69.2% (400 μ g) and 84.6% (500 μ g/ml). Acarbose showed an IC₅₀ value of μ g/ml in the alpha glucosidase inhibition assay.



- The percent inhibition of GOA III was 25.3% (100 μ g) 34.07% (200 μ g) 42.4% (300 μ g), 56.4% (400 μ g) and 72(500 μ g /ml).
- GOA III showed an IC₅₀ value of 533.04 μ g /ml in the alpha glucosidase inhibition assay.
- The percent inhibition of Acarbose was 10% (100 μ g) 46.1% (200 μ g), 53.8% (300 μ g), 69.2% (400 μ g) and 84.6% (500 μ g /ml).
- Acarbose showed an IC₅₀ value of 508.3 μ g /ml in the alpha glucosidase inhibition assay.

Fig 4: α glucosidase inhibitory activity of GOA 111

A-Amylase (EC 3.2.1.1) randomly cleaves α -(1-4) glycosidic linkages of amylase to yield dextrin, maltose, or maltotriose, whereas α -glucosidase hydrolyzes the terminal nonreducing 1-4 linked α -glucose to release glucose molecules [29]. Incubation of the α -amylase with the substrate leads to the generation of maltose; however, the addition of the drug shows significant inhibition in the liberation of

maltose in a dose-dependent manner. The analysis with GOA 111 suggested that it is a more potent inhibitor of alpha amylase and alpha glucosidase. Acarbose is a potent inhibitor of α -amylase and α -glucosidase, but adverse effects have occurred in the gastrointestinal tract because of excessive inhibition of the amylase enzyme that results in flatulence and diarrhea.

The alpha-glucosidase inhibitors also called “starch blockers” inhibit certain enzymes responsible for the breakdown of carbohydrates in the small intestine. They act mainly by decreasing the rate of carbohydrate absorption in the body. Moreover, Acarbose, an important example in this class, reversibly inhibits both pancreatic α -amylase and α -glucosidase enzymes by binding to the carbohydrate-binding region and interfering with their hydrolysis into mono-saccharides. This results in a slower absorption together with a reduction in postprandial blood- sugar levels

Uptake of glucose by yeast cells

Fig 5 and 6 depicts the effect of GOA 111 on uptake of 10mM and 25mM glucose over a 3 hour period. Results show

that the impact of the extract is as good as the standard drug at all intervals of time and for both the concentrations of glucose. When we compare the effect of the extract on the uptake of 10mM glucose over the three hours, it is observed that the effect increases appreciably from the end of the first hour to the end of two hours and sustains until the end of the third hour. But, in the case of 25 mM glucose the effect shows a sharp increase from the first hour to the second, however, declines at the end of the third hour. The results suggest that the effect of the extract is gradual yet sustained at low glucose concentration (10 mM) and sharp but short lived at higher glucose concentration (25 mM). Hence dose can be moderated depending upon the hyperglycemic levels in diabetic patients.

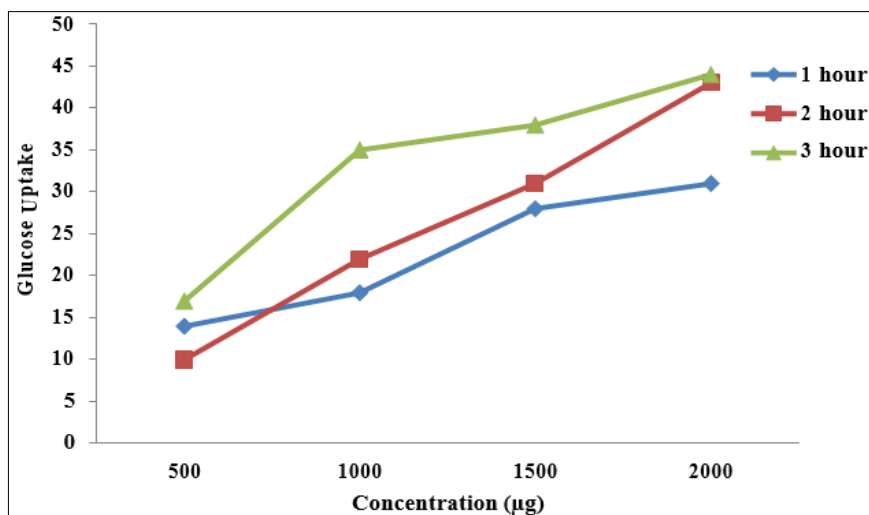


Fig 5: Effect of extract on uptake of 10mM glucose over a 3 hour period

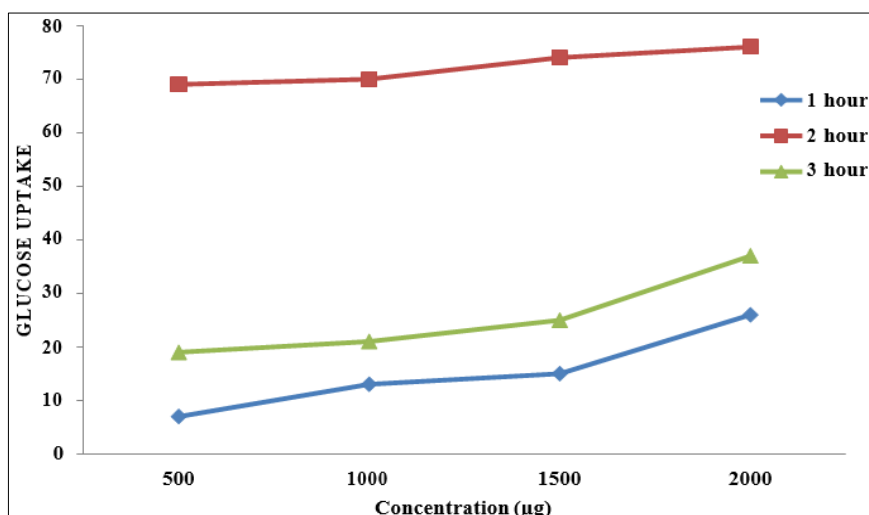


Fig 6: Effect of GOA 111 on uptake of 25mM glucose over a 3 hour period

Conclusion

The newly developed polyherbal formulation GOA 111 is rich in antioxidants and shows significant free radical scavenging potential. The results of *in vitro* antidiabetic studies reveal that GOA acts as “starch blockers”, inhibiting the activity of alpha glucosidase significantly and alpha amylase to an extent thereby controlling the release of maltose from complex carbohydrates, which may be useful in regulating glucose homeostasis. The antidiabetic evaluation also revealed that they can increase the uptake of extracellular glucose indicating a underlying mechanism causing a surge in the

secretion of insulin that needs to be investigated in further studies.

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