Sanjotha. G et al /J. Pharm. Sci. & Res. Vol. 13(10), 2021, 608-614

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Studies on Antidiabetic and anti-inflammatory properties of *Aspergillus terreus* using *in-vitro* assays

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Abstract

Aim: fungal secondary metabolites are considered to be prominent in medicinal properties due to the presences of various bioactive compounds.

Objective: objective was to study the antidiabetic and anti-inflammatory activity of *Aspergillus terreus* using *in-vitro* assays.

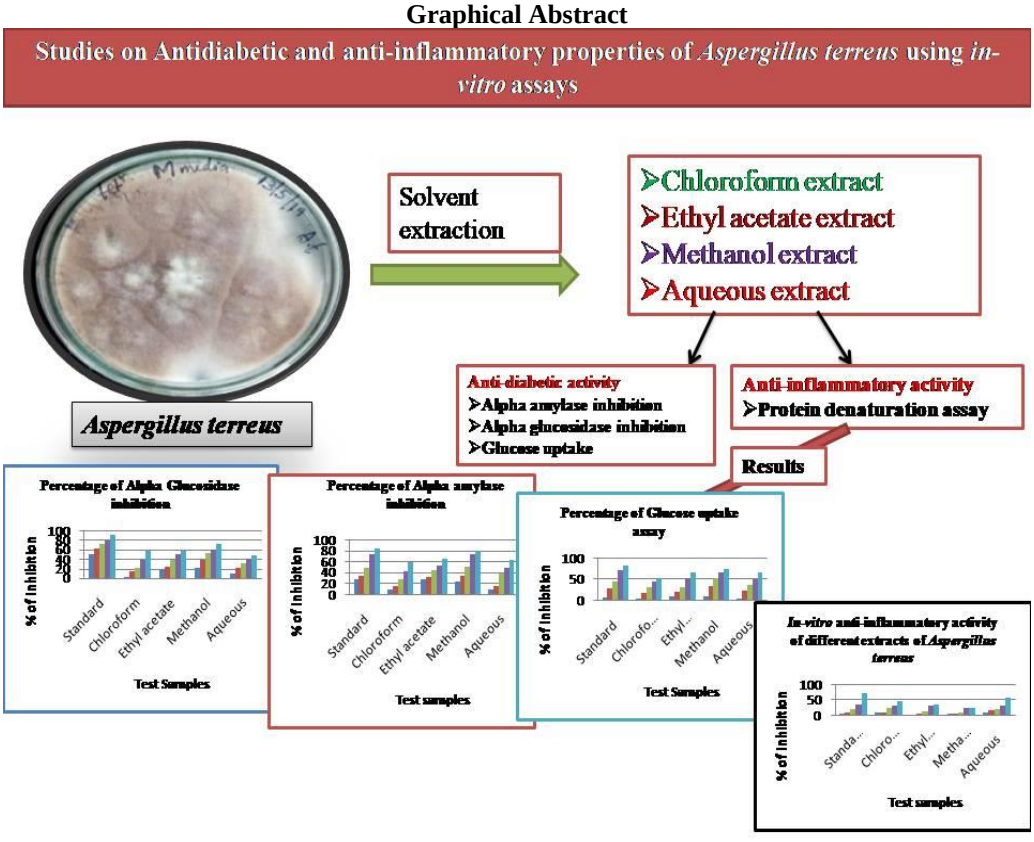
Methods: Fungal extraction was done by Soxhlet extraction method with increasing polarity of solvents viz., Chloroform, Ethyl acetate, Methanol and Water. Antidiabetic activity was evaluated by *in-vitro* alpha-amylase, alpha-glucosidase and glucose uptake assay. Whereas anti-inflammatory activity was evaluated using *in-vitro* protein denaturation assays.

Results: *In-vitro* antidiabetic activity revealed that among, the entire tested extracts methanol extract exhibited potent antidiabetic activity in all performed assays with good absorbance, percentage of inhibition and IC50 value. Anti-inflammatory studies showed that in protein denaturation assay all tested extracts shown noticeable activity with increasing in concentration but among all tested extracts aqueous extract shows significant activity with good IC_{50} value.

Conclusion: The present study revealed that different solvent extracts of *Aspergillus terreus* exhibited high antidiabetic activity and anti-inflammatory activity. Among all extracts methanol and aqueous extract shown significant activity in antidiabetic and anti-inflammatory activity respectively. Further study requires purification, Characterization and structural elucidation of compounds in aqueous extract that may help in the development of new phytopharmaceuticals.

Key words: *Aspergillus terreus*, Alpha-amylase, alpha glucosidase, Glucose uptake assay.

Graphical Abstract



INTRODUCTION

Diabetes mellitus (DM) is a well-known, progressive endocrine disorder associated with increased morbidity and mortality, as well as high health care costs. There were approximately 171 million cases of DM in 2000, and this number is expected to more than double over the next 25 years, reaching 366 million by 2030 [1, 2]. In diabetes high postprandial blood glucose leads to micro vascular complications include retinopathy, nephropathy, neuropathy, and macrovascular complications refer to increased atherosclerosis-related events such as myocardial infarction and stroke [3, 4]. Diabetes mellitus is considered as the group of disorders with different causes, which are characterized by imbalancing in carbohydrates, proteins and fat metabolism which lead to the effect on insulin action or secretion [5]. In modern medicine there is still no satisfactory effective therapy or drug to cure diabetes [6].

The currently available anti-diabetic agents include sulfonylureas, thiazolidinedione, α-glycosidase inhibitors like miglitol and acarbose widely used to control hyperglycemia but these drugs fail to cure the disease in addition causes several diabetic complications and side effects such as abdominal pain, diarrhea and soft faces in colon [7, 8]. One of the strategies adopted to treat diabetes mellitus involves inhibition of carbohydrate-digesting enzymes such as a-amylase and a-glucosidase in the gastrointestinal tract, with associated retardation of intestinal glucose absorption and lowering of postprandial blood glucose levels [9].

α-Glucosidase inhibitors fall under the third category of oral hypoglycemic agents [10]. Several α-glucosidase inhibitors, such as acarbose and voglibose obtained from natural sources, can effectively control blood glucose levels after food intake and have been used clinically in the treatment of diabetes mellitus [11]. Only a few α-glucosidase inhibitors are commercially available. All of them contain sugar moieties and their synthesis involves tedious multistep procedures. Moreover, clinically they have been associated with serious gastrointestinal side effects.Acarbose is α-glucosidase inhibitor which reduces digestion of complex carbohydrates and slows their absorption from the gut [12]. These drugs also increase the release of the glucoregulatory hormone glucagon-like peptide-1 into the circulation, which may contribute to their glucose-lowering effects. However, they may causes side effect such as malabsorption, abdominal pain, flatulence, and diarrhea which lead to a high discontinuation rate [12].

Alpha-amylase is type of the intestinal enzyme which play important role in carbohydrate digestion and glucose absorption. Suppression of the activity of digestion enzymes like α-amylase would delay the digestion of starch and oligosaccharides, which in turn decreases the absorption of glucose and consequently reduce the blood glucose [13].

Inflammation is generally referred to as a complex biological response of vascular tissues to harmful stimuli. As well, inflammation is associated with pain, and it involves in an increase ofprotein denaturation, an increase of vascular permeability, and membrane alteration, among others [14]. Inflammation is also described as the body response to inactivate or eliminate the invading stimuli or organisms, to remove the irritants and set the stage for tissue repair, and the process is accelerated by the release of chemical mediators from injured cells or tissues and migrating cells [15]. The migration of leukocytes from the venous systems to the site of damage, and the release of cytokines, are known to play a crucial role in the inflammatory response [16]. For evaluation of antidiabetic activity of drugs, *in vitro* tests can be used as initial screening tools, where the screening of large number of potential therapeutic candidates may be carried out. They might provide useful information on the mechanism of action of therapeutic agents [17].

Aspergillus terreus is a common soil saprophyte was isolated from both marine and terrestrial sources with worldwide distribution and its studies were first published in 1918 [18]. A statin drug, lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme and reductase is known for its cholesterol-lowering effect used in the treatment of heart disease and atherosclerosis, is mainly produced by *A. terreus* [19].

However there are fewer reports are available on the pharmacological studies on *Aspergillus terreus* in context to the antidiabetic and anti-inflammatory properties hence the present study was undertaken to study the antidiabetic and anti-inflammatory activities of *Aspergillus terreus.*

METHODS

Preparation of Fungal extract

About 25g of powdered biomass of *Aspergillus terreus* was extracted with 250 ml of different solvents i.e.Chloroform, Ethyl acetate, Methanol and Distilled water by using Soxhlet extraction method by marinating temperature at 50^0 C for 4 hours. The resultant extract was filtered through whatman filter paper and dried through Rota evaporator and dessicator further dried extract was stored in air tight bottle at 4°C until use.

Solvents and Reagents

All the solvents and chemicals used were analytical grade and were obtained from Hi-media, India.

Evaluation of antidiabetic activity by using *in vitro* assays

Alpha-amylase inhibitory assay

The Alpha-amylase inhibitory assay of different solvent extracts of *Aspergillus terreus* was evaluated according to a previously described method by Ranilla et al. 2008 [20]. In brief, 0.5 ml of extract was mixed with 0.5 ml of α-amylase solution (0.5 mg/ml) with 0.02 M sodium phosphate buffer (pH 6.9with 0.006MNaCl). The mixture was incubated at room temperature for 10min and 0.5 ml of starch solution (1%) in 0.02Msodium phosphate buffer (pH6.9with 0.006MNaCl) was added. The resulting mixture was incubated at room temperature for 10 min, and the reaction was terminated using 1 ml of dinitrosalicylic acid color reagent. At this time, the test tubes were placed in a water bath (100 °C for 5 min) and cooled until room temperature was attained. The mixture

was then diluted with 10 ml of deionized water, and absorbance was determined at 540 nm. The absorbance of blank (buffer instead of extract and amylase solution) and control (buffer instead of extract) samples were also determined. Acarbose was used as standard drug. The inhibition of α-amylase was calculated using the following formula :

%Inhibition of α−Amylase

=Abs Control−Abs sample/ Abs control X 100

Where Abs control corresponds to the absorbance of the solution without extract (buffer instead of extract) and with α -amylase solution and Abs sample corresponds to the solution with extract and α –amylase solution.

Glucose uptake in yeast cells

Glucose uptake assay by yeast cells was performed according to Cirillo et al., 1963 [21]. The yeast, *Saccharomyces cerevisiae* suspended in distilled water was subjected to repeated centrifugation (3000 ×g, 5 min) until clear supernatant fluids were obtained and 10% (v/v) of the suspension was prepared in distilled water. Various concentrations of different solvent extracts of *Aspergillus terreus* (50 to 250 µg/ml)were added to 1 ml of glucose solution (5mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µl of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2500 ×g, 5 min) and amount of glucose was estimated in the supernatant. Metronidazole was used as standard drug.

The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

Increase in glucose uptake

= Abs sample−Abs control/Abs sample X100

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

Alpha-Glucosidase inhibitory assay

Alpha-Glucosidase inhibition assay of α-glucosidase inhibition activity performed as described by Li et al., 2005 [22]. P-Nitrophenyl-ρ-D-glucopyranoside (PNPG), was used as substrate and prepared by dissolving in 50 mM phosphate buffer (pH 6.5). Different solvent extracts of *Aspergillus terreus* were prepared at the concentration of 50, 100, 150, 200 and 250 µg/ml dilution method (in 5% DMSO).30 µL of each concentration was added with 36 µL phosphate buffer pH 6.8 and 17 µL p-nitrophenyl-α-D-glucopiranoside (5 mM). The mixture solution was incubated for 5 min at 37°C. To this solution, 17 µL of α-glucosidase 0.15 unit/mL was added after the first incubation, and then incubated again for 15 min at 37°C. After the second incubation was finished, 100 µL of $Na_2CO_3$267 mM was added into the solution to stop the enzymatic reaction. Solution absorbance was measured with a microplate reader (BIOBASE) at 405 nm. The blank solution was tested by adding Na_2CO_3 right after the first incubation and α-glucosidase after the second incubation. Acarbose was tested as positive control.

The inhibition percentage was calculated by following formula.

Inhibition Percentage

= OD of Blank-OD of Sample/OD of Blank X 100

Statistical analysis

All experiments were performed in triplicates (n= 3) and the data are presented as the mean ± standard error. Differences between the means of the individual groups were analyzed using the analysis of variance procedure of SPSS software Version 20 (IBM).

RESULTS AND DISCUSSION

Diabetes mellitus is a clinical syndrome with severe socioeconomic importance characterized by hyperglycemias due to absolute or relative lack of insulin [23]. It is a disorder of endocrine system that is responsible for the emergence of a group of various metabolic diseases such as hyperglycemia, in which blood sugar level increases either because of insufficient secretion of insulin by pancreas or diminished response of cells towards insulin. Diabetes is classified into two types, type 1diabetes and type 2 diabetes. Numerous pathogenic responses play major roles in the development of diabetes [24]. Several management strategies have been proposed for the early stages of dysglycemia with the aim of preventing further development. The inhibition of α-glucosidase and α-amylase, key enzymes in the digestion of carbohydrates, is one way to suppress post-prandial hyperglycemia.

Alpha-amylase is the type of enzyme involved in the metabolism of carbohydrates *i.e.* hydrolysis of starch and disaccharides to glucose. In humans α-amylase expressed as two isoforms, secreted from Salivary glands and Pancreas respectively. These enzymes play an important role in digestion of polysaccharides like starch which is the main source of glucose in the human diet [25-27]. Human Salivary α-amylase (HSA) initiates the hydrolysis of α-(1, 4) glycosidic bonds in the dietary starch in to smaller oligosaccharides [28]. In the present study the different solvent extracts of *Aspergillus terreus* were subjected to α- amylase inhibitory assay along with Metronidazole as a standard. The methanol extract showed higher activity with IC 50= 152.77 µg among all other extracts tested, which was comparable to standard. The α-amylase inhibitory activities of differed solvent extracts are recorded in Table.1 and Figure.1.The inhibitors of these enzymes are considered as the potential targets in the management of diabetes mellitus.

Alpha-glucosidase this enzyme is present in the epithelial mucosa of the small intestine and cleaves the glycosidic bonds in complex carbohydrates to release absorbable monosaccharides [29]. Inhibitors of a-glucosidase display useful anti-hyperglycemic effects [30]. Alpha-glucosidase inhibitors reduce intestinal absorption of starch, dextrin, and disaccharides by inhibiting the action of α-glucosidase in the intestinal brush border. Inhibition of this enzyme slows the absorption of carbohydrates from the GI tract and decreases the rate of rise of postprandial glucose (PP hyperglycemia). This delay digestion and breakdown of

starch may have beneficial effects on insulin resistance and glycemic index control in people with diabetes [31]. In the present study, different solvent extracts of *Aspergillus terreus* were subjected to *in-vitro* alpha-glucosidase activity. The results revealed that methanol extract of *Aspergillus terreus* shown significant and promising with IC 50 value 128.85 µg. The results are shown in Table.2 and Figure.2.

Table.1. Percentage of Alpha Glucosidase inhibition

Concentration	Standard	Chloroform	Ethyl acetate	Methanol	Aqueous
50	49.56±0.000611	3.28±0.003055	17.97±0.0005033	20.99±0.0040415	8.77±0.0034395
100	60.98±0.0003606	14.98±0.00360	25.00±0.0010599	38.80±0.0004041	21.02±0.0007024
150	71.25±0.0006658	22.11±0.00513	37.91±0.0007572	52.67±0.0002517	30.46±0.0005568
200	78.54±0.0003606	38.22±0.0037859	49.56±0.0007506	59.60±0.0004041	40.79±0.0009074
250	89.49±0.0006506	56.91±0.0040501	57.11±0.000611	70.80±0.0004509	48.09±0.0004583

Results are expressed as Mean±Standard deviation

Figure.1. Percentage of Alpha Glucosidase inhibition assay

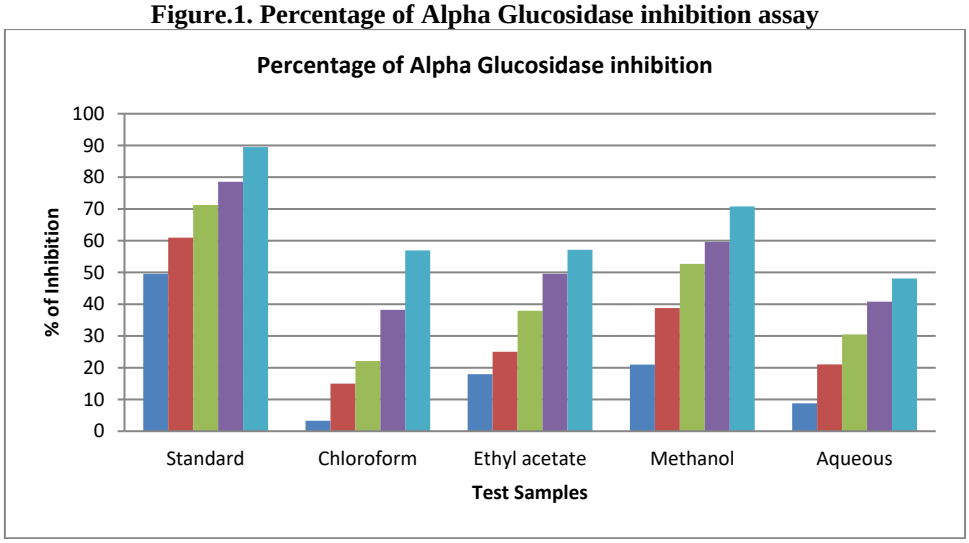
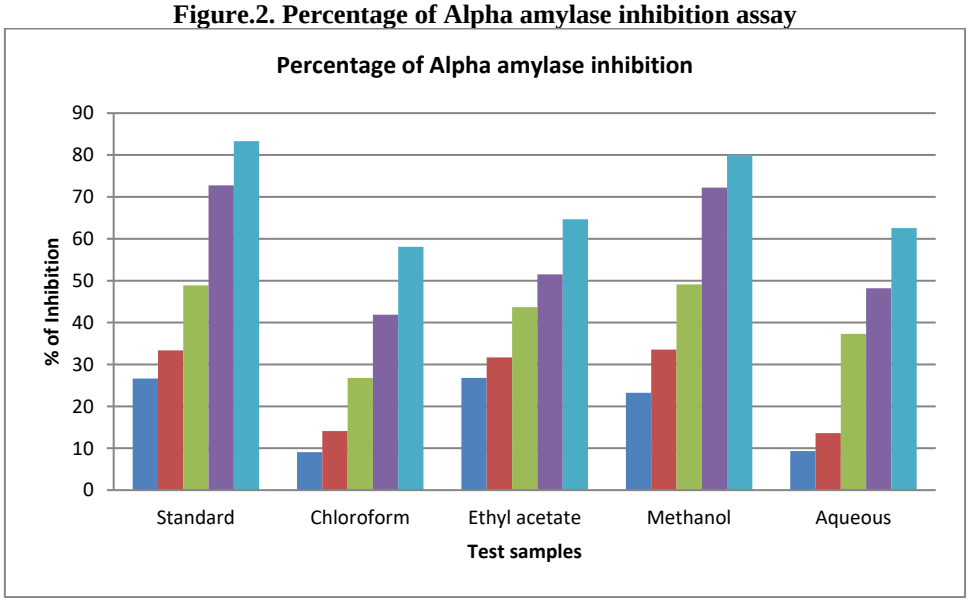


Table.2. Percentage of Alpha amylase inhibition

Concentration	Standard	Chloroform	Ethyl acetate	Methanol	Aqueous
50	26.62±0.0008083	9.06±0.0009	26.79±0.0009849	23.25±0.0009165	9.32±0.0004583
100	33.36±0.0004041	14.10±0.0006028	31.68±0.0005292	33.54±0.0007095	13.60±0.0004041
150	48.87±0.0011533	26.81±0.0009165	43.67±0.0006506	49.09±0.0006	37.30±0.0018735
200	72.75±0.0009504	41.90±0.0010017	51.52±0.0005859	72.23±0.0011	48.17±0.0007234
250	83.30±0.0010017	58.08±0.0007095	64.67±0.0003512	80.05±0.0008386	62.56±0.0004509

Results are expressed as Mean±Standard deviation

Figure.2. Percentage of Alpha amylase inhibition assay



Different concentrations of *Aspergillus terreus* solvent extracts are subjected to *in vitro* glucose uptake assay employing yeast as model. The percentage of glucose uptake in yeast cells by the extracts was compared with standard drug metronidazole. Among the all tested solvent extracts methanol extract exhibited higher activity with IC50 value 142.36 µg. There was concentration dependent increase in percentage of glucose uptake with increasing in concentration of *Aspergillus terreus*. Results also indicated that *Aspergillus terreus* had almost same efficiency in increasing the glucose uptake by yeast cells as compared to standard drug metronidazole. The results are expressed in Table.3 and Figure.3.

Inflammation is a normal protective response to tissue injury which damaged by microbial agents, physical trauma or noxious chemical. Inflammation is associated with pain, increase of vascular permeability, membrane alteration and protein denaturation due to release of lysosomal enzymes, kinins, prostroglandins and histamine [32]. The prevailing non-steroidal anti- inflammatory drugs (NSAIDs) in the treatment of diseases associated with inflammatory reactions has adverse effects which pose a major problem in the clinical use. The greatest disadvantage in the presently available potent synthetic anti-inflammatory drugs lies in their toxicity [33]. The lysosomal enzymes released during inflammation produced a variety of disorders, so stabilization of lysosomal membrane is important in limiting the inflammatory response. The search for anti-inflammatory properties has been on the rise due to their potential use in the therapy of various chronic and infectious diseases [34]. In the present study, known concentrations of different solvent extracts of *Aspergillus terreus* were subjected for anti-inflammatory activity on protein denaturation. The *in vitro* anti-inflammatory activity of the extract was comparable to the Diclofenac sodium, a reference drug. A significant difference in the inhibition of thermally induced protein denaturation was observed in tested extracts when compared with standard drug. Among the all tested solvent extracts aqueous extract shown higher activity than remaining extracts with IC50 value 21.26 µg/ml. The results are shown in Table.4 and Figure.4.

Table.3. Percentage of Glucose uptake assay

Concentration	Standard	Chloroform	Ethyl acetate	Methanol	Aqueous
50	7.89±0.0006506	6.15±0.0008505	9.75±0.0008622	10.12±0.0004509	5.54±0.0005132
100	28.80±0.0005033	19.00±0.0005	22.03±0.0005859	34.75±20.0009018	22.95±0.0004041
150	44.36±0.0005132	31.04±0.0008622	30.80±0.0002517	52.68±0.0006028	36.30±0.0008021
200	71.64±0.0004163	44.64±0.0006028	52.48±0.0004	65.14±0.0005508	49.64±0.0003055
250	82.30±0.0009609	49.30±0.0007024	66.54±0.000755	72.90±0.0005033	65.66±0.000755

Results are expressed as Mean±Standard deviation

Figure.3. Percentage of Glucose uptake assay

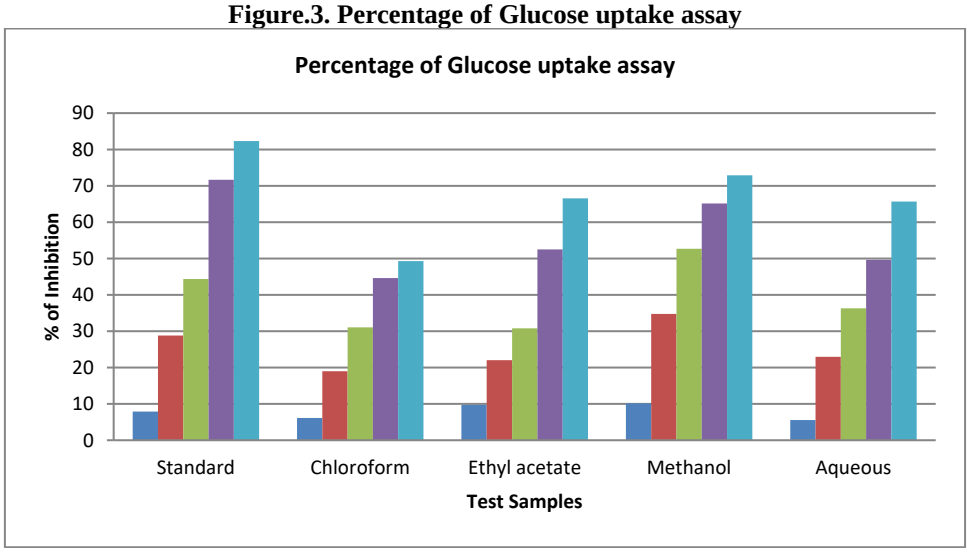
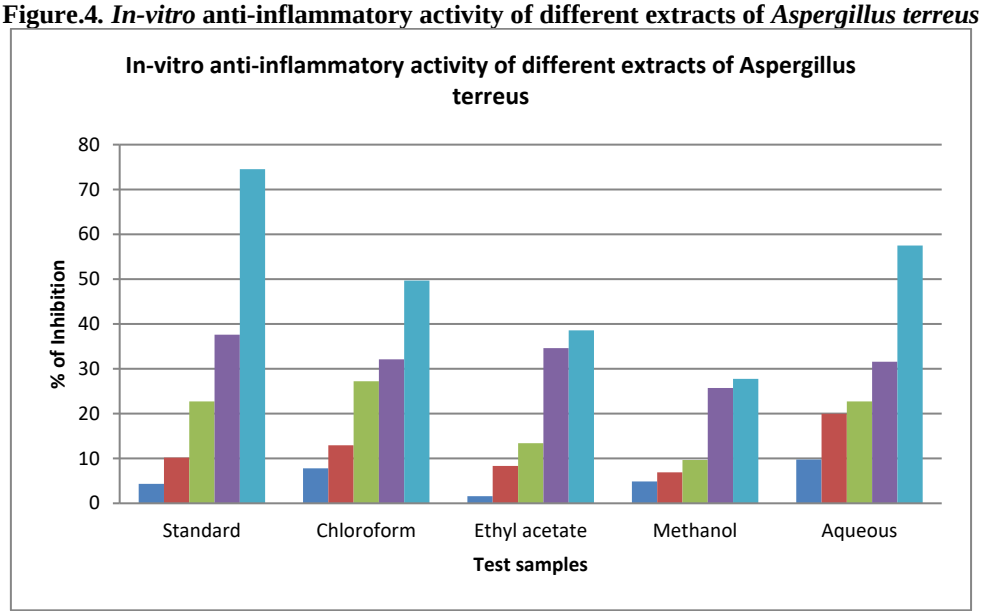


Table.4. *In-vitro* anti-inflammatory activity of different extracts of *Aspergillus terreus*

Concentration	Standard	Chloroform	Ethyl acetate	Methanol	Aqueous
50	4.34±0.022627	7.80±0.002121	1.59±0.019092	4.88±0.005657	9.76±0.003536
100	10.20±0.011314	12.95±0.002121	8.340±0.010607	6.92±0.00495	19.96±0.03677
150	22.71±0.023335	27.24±0.002828	13.39±0.004243	9.67±0.009899	22.71±0.028991
200	37.62±0.037477	32.12±0.006364	34.60±0.009192	25.73±0.00919	31.58±0.009192
250	74.53±0.002121	49.68±0.007778	38.59±0.025456	27.77±0.00707	57.49±0.026163

Results are expressed as Mean±Standard deviation

Figure.4. *In-vitro* anti-inflammatory activity of different extracts of *Aspergillus terreus*



CONCLUSION

In the present study biomass of *Aspergillus terreus* has taken and treated with different polar solvents to get different extracts. The isolated solvent extracts were subjected to *in-vitro* antidiabetic and anti-inflammatory activity. *In-vitro* antidiabetic activity revealed that it was dose dependant with the increase in the concentration the activity was increased. Among the all tested extracts methanol extract of *Aspergillus terreus* shown significant and promising activity in all performed antidiabetic assays. Whereas anti-inflammatory study revealed that all tested extracts shown noticeable activity against protein denaturation assays. In case of anti-inflammatory aqueous extract of *Aspergillus terreus* shown higher activity. Overall study concluded that methanol and aqueous extract of *Aspergillus terreus* shown significant activity in antidiabetic and anti-inflammatory activity respectively. Further studies needed on the isolation, purification and structural elucidation of compounds required for particular activity with detailed mechanism.

Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

REFERENCES

1. Funke I, Melzig MF. Traditionally used plants in diabetes therapy. Phytotherapeutics as inhibitors of a-amylase activity. RevistaBrasileira de Farmacognosia/Brazil. J. Pharm. 2006; (16): 1–5.
2. Si MM, Lou JS, Zhou CX, Shen JN, Wu HH, Yang B, He QJ, Wu HS. Insulin releasing and alpha-glucosidase inhibitory activity of ethyl acetate fraction of Acorus calamus in vitro and in vivo. J. Ethnopharmacol. 2010; (128):154–159.
3. Koda-Kimble MA. Applied Therapeutics: The Clinical Use of Drugs. 9th ed. Ch. 50. USA: Lippincott Williams & Wilkins Publisher; 2009. p. 8.
4. Pothireddy S, Chukka S, Shankaraiah P. Evaluation of antidiabetic, antidyslipidemic& hepatoprotective activity of *Homalium zeylanicum* in alloxan induced diabetic rats. Int J Res Dev Pharm Life Sci. 2014; 3(3):1004-10.
5. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care 2007; 30: S42-S46.
6. Ali H, Houghton PJ, Soumyanath A. Journal of Ethnopharmacology 2006; 107: 449–455.
7. Kokiwar PR, Guptha S, Durge PM. Prevalance of diabetes in a rural area of central India. Int J Diab Dev Ctries. 2007; 27(1):8-10.
8. Mohan V, Sandeep S, Deepa R, Shah B, Varghese C. Epidemiology of Type 2 diabetes: Indian scenario. Indian J Med Res. 2007; 125(3):217-30.
9. Rhabasa-Lhoret R, Chiasson JL. Alpha-glucosidase inhibitors. In: Defronzo, R.A., Ferrannini, E., Keen, H., Zimmet, P. (Eds.), International Textbook of Diabetes Mellitus, 2004;3rd Ed., vol. 1. John Wiley, UK.
10. Du WQ, Shi XF, Qiu MY et al. Progress in treatment of diabetes drugs, Chin. Hosp. Pharm. J. 2005; 25 ,67–69.
11. Playford RJ, Pither C, Gao R, et al. Use of the α-glucosidase inhibitor acarbose in patients with 'Middleton syndrome': normal gastric anatomy but with accelerated gastric emptying causing postprandial reactive hypoglycemia and diarrhea, Can. J. Gastroenterol. 2013; 27: 403–404.
12. Bennett PN, Brown MJ. Clinical Pharmacology. 11th ed. Edinburgh: Churchill Livingstone Elsevier Publisher; 2012. p. 580.
13. Vadivu, Rajendran, and K. S. Lakshmi. "In vitro and in vivo anti-inflammatory activity of leaves of Symplocos cochinchinensis (Lour) Moore ssp Laurina." ||| Bangladesh Journal of Pharmacology||| 3.2 (2008): 121-124.
14. Ferrero-Millani L, Nelsen OH, Anderson PS, Girardin SE. Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1 beta generation. Clin. Exp. Immunol. **2007**; 147: 227–235.
15. Chandra S, Chatterjee P, Dey P, Bhattacharya S. Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of the protein. Asian Pac. J. Trop. Biomed. **2012**; 2: 178–180.
16. Hollman PCH. Absorption, bioavailability and metabolism of flavonoids. Pharm. Biol. 2004; 42, 74–83.
17. Roy A, Geetha RV. *In vitro* α-amylase and α-glucosidase inhibitory activities of the ethanolic extract of *dioscoreavillosa*tubers. Int J Pharm Bio Sci. 2013;4(4):49-54.
18. Wang Y, Zheng J, Liu P, Wang W, & Zhu W. Three compounds from *Aspergillus terreus* PT06-2 grown in a high salt medium. *Marine Drugs* 2011; 9: 1368-1378.
19. Hajjaj H, Peter N, & Philippe D.L ovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. *Journal Applied and Environment Microbiology*.2001;67(6): 2596-2602.
20. Ranilla LG, Young-In G, Maria Ines L, Franco M, Shetty K. Antidiabetes and antihypertension potential of commonly consumed carbohydrate sweeteners using in vitro models. Journal of Medicinal Food. 2008; 11: 337–348.

21. Cirillo VP, Wilkins PO, Anton J. Sugar transport in a psychrophilic yeast. Journal of Bacteriology. 1963;86: 1259–1264.
22. Li T, Zhang XD, Song YW. A microplate-based screening method for alphaglucosidase inhibitors. Chinese Chin J Clin Phamaco Ther. 2005;10:1128–34.
23. Aguwa CN. Therapeutic Basis for Clinical Pharmacy in the Tropics, 3rd ed. SNAAP Press Ltd., Enugu, pp. 2004; 125–230.
24. Bhutkar M &Bhise S. *In vitro* assay of alpha amylase inhibitory activity of some indigenous plants. *Int J Chem Sci*. 2012; 10(1): 457-462.
25. Qin X, Ren L, Yang X, Bai F, Wang L, Geng P, Bai G, Shen Y: J. Struct. Biol 2011; 174, 196.
26. Butterworth PJ, Warren FJ, Ellis PR: Starch-Starke 2011; 63: 395.
27. Groot PC, Bleeker MJ, Pronk JC, Arwert F, Mager WH, Planta RJ, Eriksson AW, Frants RR: Genomics 1989; 5: 29.
28. Nikitkova AE, Haase EM, Scannapieco FA: Appl. Environ. Microbiol 2013; 79: 416.
29. Chiba S. Molecular mechanism in a-glucosidase and glucoamylase. Bio. Sci. Biotech. Biochem. 1997; 61, 1233–1239.
30. Stuart, A.R., Gulve, E.A., Wang, M., Chemistry and biochemistry of type 2 diabetes. Chem. Rev. 2004; 104, 1255–1282.
31. Uddin N, Hasan MR, Hossain MM, Sarker A, Hasan AH, Islam AF, et al. *In vitro* α-amylase inhibitory activity and *in vivo* hypoglycemic effect of methanol extract of *Citrus macroptera*Montr. Fruit Asian Pac J Trop Biomed. 2014;4(6):473-9.
32. Leelaprakash G, Mohan DS. *In vitro* Anti- Inflammatory activity of methanol extract of *Enicostemmaaxillare*. Int J Drug Develop Res. 2011; 3(3): 189-196.
33. Chawla AS, Handa SS, Sharma AK, Kalth. Plant anti-inflammatory agents. J Scient Ind Res. 1987; 46: 214-223.
34. Halliwell B. Antioxidants in human health and disease. Annu Rev Nutr. 1996; 6: 33-50.