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ORIGINAL ARTICLE

In Vitro Evaluation of Antioxidant and Antidiabetic Potential of Poly Herbal Formulation

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ABSTRACT
Ethno medicines utilize phytochemicals in the formulation of drugs with less or no side effects. In this present investigation, a polyherbal formulation (PHF) was prepared using Catharanthus roseus, Cassia auriculata and Sauropusa bdrogynus for evaluation of its antioxidant and antidiabetic efficacy using in vitro models. The identification of bioactive compounds present in the ethanolic extract of PHF was carried out using GC-MS analysis. The antioxidant activity was determined using DPPH radical scavenging assay. The antidiabetic activity was assessed by α-amylase and α-glucosidase enzyme inhibitory activity of PHF. 9,12-Octadecadienoic acid (53.47%), Oleic acid (12.76) and ethyl linoleolate (12.40%) were the major compounds identified in prepared PHF. Further, the PHF exhibited effective DPPH radical scavenging activity and also possessed significant inhibitory activity against enzyme, α-amylase and α-glucosidase with the IC_{50} of 30.99 µg/ml and 38.72 µg/ml respectively. The findings of this study,suggest that the developed PHF could be an ideal herbal combination for the management of hyperglycemia and oxidative stress.
Key Words: *Antioxidants, Catharanthus roseus, Cassia auriculata, Diabetic mellitus, Polyherbal formulation*

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INTRODUCTION

Diabetes Mellitus (DM) is a complex metabolic disorderliness due to post prandial hyperglycemia [1]. Diabetic patients' accounts to 463 million world-wide and 77 million are Indians. DM among Indian population has rapidly increased to 8.9% and 134 million people will be diabetic by the end of 2045 [2]. According to the report of WHO, DM accounts for 2% of death in India [3]. Hyperglycemia increases the generation of reactive oxygen species (ROS) that has negative regulation on insulin signaling pathway leading to mitochondrial and β-cell dysfunction, impaired glucose tolerance and resistance to insulin response [4]. Excessive production of ROS leads to oxidative stress induced cell damage and diabetic complexity [5]. The two most important enzymes that are responsible for hyperglycemia are α-amylase and α-glucosidase. α-amylase, hydrolysethe complex carbohydrates (Polysaccharides) into oligosaccharides, while α-glucosidase breaks down the oligosaccharides into simple sugars. The therapeutic intervention currently employed is to inhibit these enzymes. Acarbase, a natural product of Actinoplanes strain and miglitol, a natural bioactive compound from *Morus* species are the α-glucosidase inhibitor drugs [6]. The limitations of these inhibitory drugs are its secondary responses such as hypoglycaemia and gastro-intestinal abnormalities [7]. Previous reports proved that excessive ROS generation induced diabetic complications can be treated or prevented with sufficient supply of antioxidants [8]. Diet rich in plant derived materials rectify the DM associated complications [9]. Thus antioxidants from plant and plant products serve as safe alternative in treating DM [8]. Developing countries like India depend on Ayruvedic, Siddha and Unani utilizes plant and plant products for the formulation of drugs [10]. The pharmacological properties are because of the phytochemicals present (or) extracted from plants.

The polyhedral formulation (PHF) contains bioactive compounds from plant sources that acts synergistically in enhancing the therapeutic potential [11] and hence advantageous over the single

compound drugs [12]. The polyherbal therapies (herb-herb combination) have been in practice before several decades in China and the concept of drug combination proved remarkable success of western medicine in treating various diseases [13]. The polyherbal formulations are advantageous because of its nature obtained ingredients and its potential interaction effects such as mutual enhancement, assistance and resistance [14]. Ayruvedic formulations, Bharangyadi [15] and Indukantha Ghritha is being in practice for treating several kinds of diseases. In Unani medicine, a polyherbal formulation known as Majoon Surnjan is prepared with 17 herbal combinations for treating rheumatoid arthiritis. Alcoholic extract of *Holarrhena antidysentrica*, *Prunusdulcis*, *Cicer arietinum* and oleic acid combination was found effective in treating type II diabetes [16]. Rahim *et al.,* (2018) reported the antioxidant activity of polyherbal drug consisting of *Strobilanthescrispus*, *Phyllanthus niruri*, *Orthosiphona ristatus* and *Stevia rebudiana*by DPPH assay. Herbal formulation of *Azadirachta indica,Ocimum sanctum* and *Gymnemasylvestre*, (1:1:1 ratio) showed effective antioxidant and antidiabetic potentials [17]. The polyherbal drug BGR-34 consisting of *Berberisaristata*, *Tinosporacordifolia*, *Trigonellafoenum-graecu*, *Pterocarpus marsupium*, *Gymnema sylvestre* and *Rubiacordifolia*, inhibited hyperglycemic causative enzymes [10].

In this context, a poly herbal formulation, designated as ADOJ was developed using leaves and flowers of *Catharanthusroseus* and *Cassia auriculata* and leaves of *Sauropusabdrogynus*. *Catharanthusroseus*is an exigent herb in traditional medicine system of China, India, South Africa, Malaysia [18] and Mexico in treating diabetes [19]. The flowers and leaves extract of *Cassia auriculata* has beneficial effects against skin diseases diabetes[20] and to reduce body heat [21]. Further, *Cassia auriculata*is an excellent antihyperlipidaemic, antioxidant and antidiabetic agent [22]. The flavonoids and phenolic compounds of leaves of *Sauropus androgynous* are antioxidants[23]and antidiabetic [24]. Hence the present research aims to develop a polyherbal formulation (ADOJ), to determine the synergistic effect of bioactive principles present in *Catharanthus roseus*, *Cassia auriculata* and *Sauropusabdrogynus* for its antioxidant and antidiabetic activities.

MATERIAL AND METHODS

Chemicals and reagents

Chemicals such as ethanol, starch, α-amylase, α-glucosidase, 3,5-dinitrosalicylic acid (DNSA), Para-nitrophenyl α-D-glucopyranoside (p-NPG), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Himedia Laboratories, Mumbai, India. Standards including ascorbic acid and ascorbate were purchased from Sigma-Aldrich, USA. Reagents and chemicals used were of analytical grade.

Collection and authentication of plant materials

The plants (*Catharanthu sroseus, Cassia auriculata, Sauropus androgynous*) forpolyherbal formulation were collected in and around Thanjavur, Tamil nadu, India and were authenticated by the Department of Plant Science, Bharathidasan University, Thiruchirapalli, India.

Preparation of Polyherbal formulation (ADOJ)

Polyherbal formulation (PHF) consists of dried plant powders of *Catharanthusroseus*(leaves and flowers), *Cassia auriculata* (leaves and flowers) and *Sauropus androgynous* (leaves). After collection, the plants materials were washed and shade dried. After 15 days, the polyherbals were pulverized individually into fine powder. Each parts of dried powder were weighed (20g) accurately and mixed together. Finally, it was stored in airtight container and used for further analysis.

Plant extraction

Ethanol extracts (1:5 w/v) of the pulverized samples (100 g) were obtained using mechanical shaker for 72 h. The extract was filtered and concentrated under reduced pressure to yield crude extract.

Gas Chromatography Mass Spectroscopy Analysis

Bioactive compounds of the formulated PHF-ADOJ were identified with GC-MS analysis (QP 2010 PLUS, SHIMADZU, JAPAN). Fused silica column packed with Elite -5MS (5% biphenyl 95% dimethylpolysiloxane) and carrier gas used was helium (flow rate 1ml/min) for the separation of components. The temperature of the injector was adjusted to 260^{o}C during chromatographic run. 1 ml of PHF-ADOJ was introduced and the oven was set at 60^{o}C for 2 min and increased constantly to 300^{o}C with 10^{o}C rise for every 10 min. and finally fixed at 300^{o}C for 6 min. The interpretation of GC-MS spectrum was donewith National institute of Standard and Technology for the identification of bioactive compounds.

In vitro antidiabetic activity

Percentage inhibition of the enzymes (α-amylase and α-glucosidase) by ADOJ were carried out in DMSO by previously optimized procedure [24]. Standard drug, Acarbose was served as positive control for calculating IC_{50}values.

α -amylase inhibitory assay

For the preparation of 1% starch solution 1g starch was diluted in 100 ml of 20 mM of phosphate buffer (pH 6.9) containing 6.7mM of sodium chloride. 27.5 mg of pancreatic α-amylase was added to 100 ml of phosphate buffer (PBS, 20 mM, pH 6.9) containing sodium chloride (6.7 mM) for the preparation of enzyme solution. To 100 µl of various concentrations of PHF extract (10, 20, 30,40, 50 µg/ml) 200 µlof pancreatic amylase was added and the mixture was incubated at 37 ºc for 20 min. To this, 100 µl (1%) starch solution was added and incubated at 37 ºC for 10 min. 200 µl DNSA (1g of 3,5 di nitro salicylic acid, 30g of sodium potassium tartarate and 20 ml of 2N sodium hydroxide was added and made up to a final volume of 100 ml with distilled water)was added to complete the reaction and kept it in a boiling water bath for 5 minutes. 2.2 ml of water was added to dilute the sample and the absorbance of the prepared mixture was read calorimetrically at 540 nm . Blank was setup with 200 µL of dilute water replacing enzyme solution. Similarly control was prepared without extract representing 100% enzyme activity. The assay was performed in triplicates.

α -Glucosidase Inhibition Assay

α-glucosidase (1mg) was dissolved in 100 ml of phosphate buffer (pH 6.8) and 200 µl of this enzyme was addedeto 100 µl of various concentrations of PHF extract (10, 20, 30,40, 50 µg/ml) and the mixture was incubated at 37ºC for 20 min. To the reaction mixture 100 µlof 3mM p-nitrophenyl –D glucopyranoside (p-NPG) was added and incubated at 37 ºC for 10 min. The reaction was terminated with 2ml Na_2CO_3(0.1M) and the α-glucosidase activity was determined spectrophotometerically at 405 nm (Shimadzu UV-1800) by measuring the quantity of p-nitrophenol released from p-NPG. Acarbose was the positive control. The concentration of the extract required to inhibit 50% of α-amylase and α-glucosidase activity under the assay conditions was defined as the IC_{50} value. IC_{50} was calculated by

% Inhibition = $(A_c-A_s)/A_c$ X 100

where A_c= absorbance of the control and A_s= absorbance of the sample.

DPPH scavenging activity

DPPH scavenging activity of the extract was determined by the method of Jain and Agarwal [25]. Several concentrations of PHF extract (10, 20, 30l, 40 & 50µl)) was added separately to 50 µl of DPPH (0.659 mM) solution and incubated at 25°C for 20 min. The absorbance was measured at 510 nm using shimadzu UV 1800 spectrophotometer. Same procedure used for control without sample. Ascorbic acid served as the positive control. The % inhibition was calculated by the equation

% Inhibition = $(A_0-A_t)/A_0$ X 100

where A_0= absorbance of the control (blank, without extract) and A_t= absorbance of the extract.

Data analysis

The values are represented as Mean±SD in triplicates. IBM statistical package, SPSS (Version 23) was used to calculate IC_{50} value.

RESULTS AND DISCUSSION

DM, is a complex metabolic disorder that has an increasing trend in occurrence all over the world. Several types of oral hypoglycaemic drugs and insulin are available and effectively used in the treatment of DM. Besides these conventional diabetic medications, renewed interest in using natural products in treating diabetes is getting paramount importance because of its limited side effects [26]. PHF are advantageous besides single herbal therapy since it exhibit expanded remedial potential. Hence the present investigation utilizes different herbal combinations in equal proportion for the evaluation of its antioxidant and antidiabetic properties [10]. The identification of bioactive compounds present in the polyherbal formulation was carried out using GC-MS analysis. Twenty different bioactive compounds were identified and shown in the table (1) and the GC-MS chromatogram was presented in figure (1).

9,12-Octadecadienoic acid (53.47%), Oleic acid (12.76) and ethyl linoleolate (12.40%) were the major compounds identified. 9,12-Octadecadienoic acid is a polyunsaturated omega-6 fatty acid that has potential antioxidant and antidiabetic activity [27]. 9,12,15-octadecatrien-1-ol, a long chain fatty alcohol is an inhibitor of protein- tyrosine phosphatase 1 B [28]. Hwang *et al*., [8] reported that hexadecanoic acid, ethyl palmitate, 9,12,15-Octadecatrienoic acid and ethyl linoleolate obtained from ethanol extract of *Prunella vulgaris* exhibited significant antioxidant and anticancer activities. Hexadecanoic acid, methyl ester a fatty acid methyl ester and hexadecanoic acid, possess antidiabetic activity [28] as well as antioxidant activity [29]. Pentadecane, a saturated fatty acid identified in the polyherbal formulation is a sugar phosphate inhibitor. Similarly, 2,4-decadienal, a polyunsaturated fatty aldehyde is an apoptosis inducer [4]. Thus the presence of these phytochemicals may arguably responsible for the therapeutic effect of the polyherbal formulation. Further none of the identified phytochemicals has alarming toxicity

effects. Hence the polyherbal formulation prepared in the present study is within the safety profile of herbal formulation.

Figure 1: GC-MS Chromatogram of PHF-ADOJ

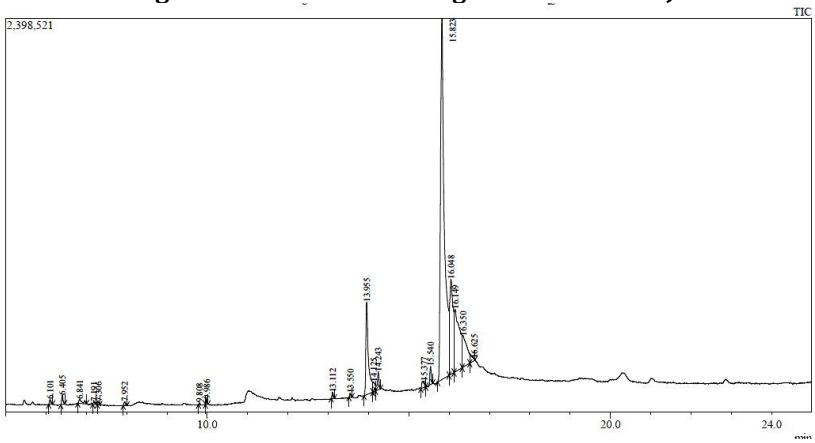


Table 1: Bioactive compounds identification from PHF-ADOJ by GC-MS analysis

Name of the Compound	Molecular Formula	Retention time	Area %
1,5-Hexadiene, 2-methyl-	C_7H_{12}	6.101	0.26
2,4-Decadienal, (E,E)-	$C_{10}H_{16}O$	6.405	0.51
Octane, 3,4,5,6-tetramethyl-	C_7H_{12}	6.841	0.51
4-Heptenal	$C_{12}H_{26}$	7.191	0.14
Octane, 2,3,3-trimethyl-	$C_{11}H_{24}$	7.306	0.13
trans-Caryophyllene	$C_{15}H_{24}$	7.952	0.21
Dihexylsulfide	$C_{12}H_{26}S$	9.808	0.01
2-Nitrobenzaldehyde	$C_7H_5NO_3$	9.986	0.24
Bis (2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	13.112	0.29
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13.550	0.17
9-Octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	13.955	9.06
1,4-Cyclohexanediol, dibenzoate	$C_6H_{12}O_2$	14.125	0.70
Pentadecanoic acid, ethyl ester	$C_{17}H_{34}O_2$	14.243	1.22
9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O$	15.377	0.66
Phytol	$C_{20}H_{40}O$	15.540	0.88
9,12-Octadecadienoic acid (Z,Z)	$C_{18}H_{32}O_2$	15.823	53.74
9-Octadecenoic acid (Z)	$C_{18}H_{34}O_2$	16.048	12.76
Ethyl linoleolate	$C_{20}H_{34}O_2$	16.149	12.40
Propionic acid, 3-(2-methylcyclohexyl) -, ethylester	$C_{12}H_{22}O_2$	16.350	5.36
3-Octanol, 1-bromo-1,1,2,2-tetrafluoro-	$C_8H_{13}BrF_4O$	16.625	0.75

DPPH Radical Scavenging Activity

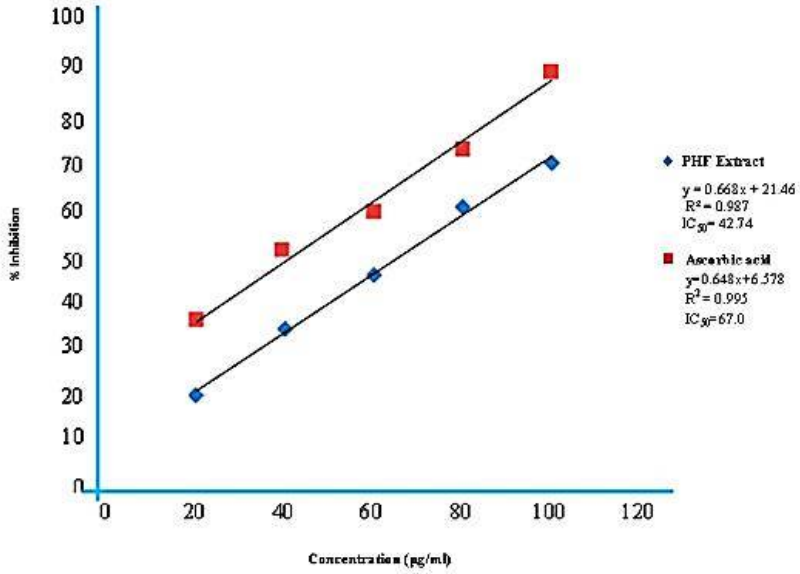
Balance between the generation and elimination of free radicals plays a critical role in development of oxidative stress and several other complications [30]. In the present investigation the antioxidant property of PHF-ADOJ was assessed using DPPH assay [31]. DPPH is a nitrogen focused free radical that receives either hydrogen or electrons from the antioxidant compounds present in PHF. Fig (2) presents the DPPH scavenging activity of the PHF-ADOJ. The DPPH radical scavenging activities of PHF at different concentrations (20, 40, 60, 80 and 100 µg/ml) were shown in Table (2) and were compared with the reference drug, ascorbic acid. The free radical scavenging activity of PHF-ADOJ1 was concentration dependent and was found to be 18.64% (20µg/ml), 33.32% (40µg/ml), 45.20% (60µg/ml), 60.34% (80µg/ml) and 70% (20µg/ml).

Table 2: DPPH radical scavenging activity of PHF-ADOJ and Ascorbic acid

Concentration (µg/ml)	% of Inhibition	
	PHF extract	Ascorbic acid (Standard)
20	18.64±0.12	34.22±0.44
40	33.32±0.72	51.20±0.42
60	45.20±0.14	59.20±0.18
80	60.34±0.16	73.0±0.82
100	70.0±0.92	90.12±0.88
IC_{50}	67.0±0.82	42.74±0.54

The IC_{50} value (67.0µg/ml) of PHF-ADOJ was low compared to the standard, ascorbic acid (42.74µg/ml). The PHF-ADOJ used in the present study exhibited better DPPH radical scavenging activity (IC_{50}=67.0µg/ml) than the PHF, GOA 111 (IC_{50}=633.800µg/ml). U [20] ddandrao*et al*., [31] reported the 20% inhibition of DPPH radical at 50.74µg/ml concentration with PHF prepared using *Piper nigrum*, *Terminalia paniculata* and *Bauhinia purpurea.* The possible reason for high DPPH radical scavenging activity in the present study might be attributed to the difference in bioactive compounds.

Figure 2: DPPH radical scavenging activity of PHF-ADOJ and Ascorbic acid



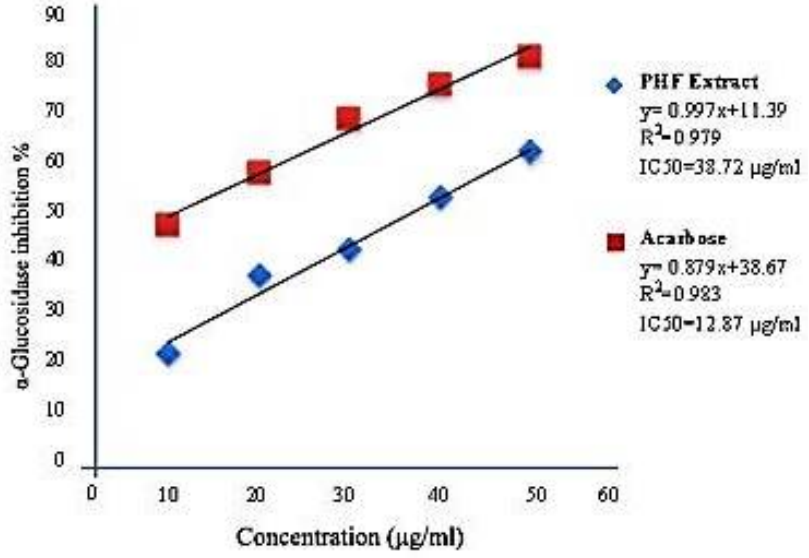
Inhibition of α-amylase and α-glucosidase

The enzyme α-amylase cleaves α-(1-4) glycoside bonds present in amylose to produce oligosaccharides such as maltose, dextrin etc. Similarly, the α-glucosidase enzyme is involved in the hydrolysis of terminal α-(1-4) linked glucose molecule [32]. But the addition of PHF-ADOJ inhibit the generation of oligosaccharides from complex poly saccharides. However, significant inhibitory effect was observed with PHF-ADOJ against α-amylase (IC_{50}=30.99 µg/ml), it was comparatively low to the inhibitory effect (IC_{50}=16.89 µg/ml) of the standard drug, ascorbase used in present investigation (Figure 3). Similarly, α-glucosidase inhibition by PHF-ADOJ (Figure 4) was also found to be low (IC_{50}=38.72 µg/ml) compared to the standard drug (IC_{50}=12.87 µg/ml). Though, ascorbase exhibited potential inhibitory activity against both α-amylase and α-glucosidase severe adverse effects such as flatulence, diarrhea [33], hepatitis and increase in level of liver enzymes are reported [34]. Hence the new PHF would be a safe alternative in the treatment of diabetes mellitus that shows fewer side effects due to the presence of naturally derived phytochemicals. [35]

Table 3: IC_{50} values of standard drug acarbose and PHF-ADOJ against α-amylase and α-glucosidase

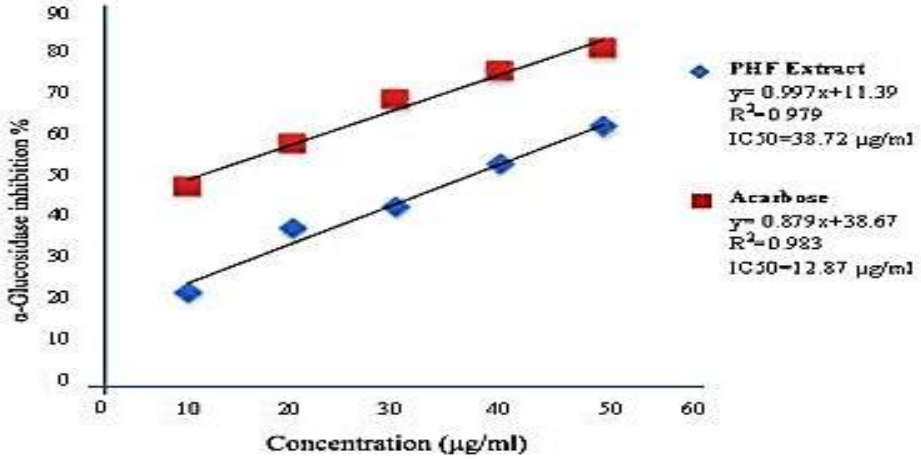
Sample	IC_{50} (µg/ml)	
	α-amylase	α-glucosidase
PHF extract	30.99±0.80	38.72±0.52
Acarbose	16.89±0.76	12.87±0.26

Figure 3: Inhibitory activity of PHF –ADOJ and standard drug Acarbose on α- Amylase



PHF designated as ADJ which includes *Momordica, Psidiumguajava, Phyllanthusembica, Trigonellafoenum-graecum, Syzygiumcuminii*and *Gymnemasylvestre* exhibited significant α-amylase and α-glucosidase inhibitory activity with IC50 values of 0.41±0.03 mg/ml and 0.51±0.01mg/ml respectively. But the inhibitory activity exhibited by the PHF was low compared to the standard drug, ascorbase that showed IC50 values of 0.2±0.01 and 0.39±0.02 mg/ml for α-amylase and α-glucosidase respectively. The results of this present investigation were in agreement with the antidiabetic activity of PHF-ADJ [36].

Figure 4: Inhibitory activity of PHF –ADOJ1 and standard drug Acarbose on α- glucosidase



CONCLUSION

The PHF prepared in this study contained high amount of bioactive compounds that contributed to its therapeutical potential. These bioactive compounds derived from *Catharanthus roseus*, *Cassia auriculata*and *Sauropusa bdrogynus* exhibited effective free radical scavenging as well as inhibitory activity against α-amylase and α-glucosidase. Hence these biological activities of PHF-ADOJ might be explored further in the management of DM. Further, the study also proposed the idea of herbal combination in the treatment of DM over single herbal therapy. The synergistic activity of the bioactive compounds from different plant sources enhanced the antioxidant and antidiabetic efficacy of the PHF. *In vivo* investigation on the antidiabetic potential of the PHF-ADOJ needs to be explored in future.

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CONFLICTS OF INTEREST

Author have no conflicts of interest.

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