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IN-VITRO ANTIOXIDANT AND ANTIDIABETIC ACTIVITY OF THE COMBINED S-ALLYL CYSTEINE AND TAURINE

Nadeem Rais [*1], Akash Ved [2], Rizwan Ahmad [3], Kehkashan Parveen [4] and Mohd. Mujeeb [5]

Department of Pharmacy [1], Bhagwant University, Ajmer – 305004, Rajasthan, India.
Goel Institute of Pharmaceutical Sciences [2], Faizabad Road, Lucknow - 226028, Uttar Pradesh, India.
Department of Pharmacy [3], Vivek College of Technical Education, Bijnor - 246701, Uttar Pradesh, India
Interdisciplinary Biotechnology Unit [4], Aligarh Muslim University, Aligarh - 202001, Uttar Pradesh, India
Department of Pharmacognosy and Phytochemistry [5], Faculty of Pharmacy, Jamia Hamdard, New Delhi - 110062, Delhi, India.

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Correspondence to Author:
Mr. Nadeem Rais

Research Scholar,
Department of Pharmacy,
Bhagwant University, Ajmer – 305004, Rajasthan, India.

E-mail: drx.nadeemrais@gmail.com

ABSTRACT: Diabetes mellitus is one of the familiar metabolic disorders affecting the global population. In the modern system of medicine, there are still no satisfactory promising agents available for the effective management and treatment of diabetes. The current study aimed to investigate the in-vitro antioxidant and antidiabetic potential of the combination of s-allyl cysteine (a phytochemical) and taurine (an endogenous biomolecule). Despite numerous pharmacotherapeutic properties of s-allyl cysteine (SAC) and taurine (TAU), their combined *in-vitro* effects as antidiabetic and antioxidant had not been explored, and scientific data were also lacking to be exposed as yet. The combination (SAC/TAU) gives in-vitro antioxidant activity when subjected to the tests like reducing power, hydrogen peroxide & DPPH radical scavenging assays and *in-vitro* antidiabetic activity when subjected to the tests like α-glucosidase & α-amylase inhibitory activities. The IC_{50} value were reducing power test (significant with the ascorbic acid), hydrogen peroxide radical scavenging test (4.84 µg/ml), DPPH assay (16.30 µg/ml), α-amylase inhibitory activity test (17.26 µg/ml), and α-glucosidase inhibitory activity test (5.73 µg/ml). Hence, the *in-vitro* antioxidant and antidiabetic results indicate the potential of the combination (SAC/TAU) to manage hyperglycemia and related complications.

INTRODUCTION: Oxygen is essential for the survival of all on this earth. Oxygen may be toxic when it leads to the generation of free radicals (FR) such as superoxide ($\bullet O^{2-}$), hydroxyl (•OH), singlet oxygen ($1O_2$) and other secondary reactive oxygen species, giving rise to a chain of molecular alterations consisting, in the early stages, of lipid peroxidation products [1].

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During the utilization and metabolism of oxygen, approximately 5% of oxygen gets univalent reduced to oxygen-derived free radicals such as superoxide, hydrogen peroxide, hydroxyl, peroxynitrite, and nitric oxide radicals.

All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the human body cells, rendering each cell face about 10,000 oxidative hits per second [2]. Several diseases are caused by oxidative stress, which results from an imbalance between the formation and neutralization of pro-oxidants [3, 4]. The natural antioxidants act as potent free-radical scavengers, reducing agents, potential complexes of prooxidant

metals, quench of singlet oxygen *etc.* [5] the antioxidants can interfere with the oxidation process by reacting with free radicals [6, 7]. In general, the effect of antioxidants is to discontinue the chain formation during the propagation process by providing a hydrogen atom or an electron to the free radical and receiving the excess energy possessed by the activated molecule [8]. Oxidative stress, initiated by free radicals, plays a vital role in damaging various cellular macromolecules. This damage may result in many diseases, including nephrotoxicity, diabetes mellitus (DM), atherosclerosis, myocardial infarction, arthritis, anemia, asthma, inflammation, neurodegenerative diseases, and carcinogenesis [9]. Treatment of Type 2 diabetes mellitus (T2DM) remains a challenging issue. During the last decade, there has been much interest in the development of anti-diabetic drugs [10, 11]. Increasing evidence suggests that oxidative stress is a prominent and early feature in the pathogenesis of diabetes mellitus due to persistent hyperglycemia [12]. Therefore a therapeutic approach to treat diabetes is to decrease postprandial hyperglycemia [13]. This can be achieved by the inhibition of carbohydrate hydrolyzing enzymes like alpha-amylase and alpha-glucosidase [14]. Alpha-glucosidase and alpha-amylase are the important enzymes involved in the digestion of carbohydrates. Alpha-Amylase is involved in the breakdown of long-chain carbohydrates, and alpha-glucosidase breaks down starch and disaccharides to glucose.

They serve as the major digestive enzymes and help in intestinal absorption. Alpha-amylase and glucosidase inhibitors are the potential targets in the development of lead compounds for the treatment of diabetes [15, 16]. Some inhibitors currently in clinical use are acarbose, miglitol, and voglibose are known to inhibit a wide range of glycosidases such as α-glucosidase and α-amylase. Because of their nonspecificity in targeting different glycosidases, these hypoglycemic agents have their limitations and are known to produce serious side effects [17]. However, clinical trials have been disappointing as, so far, they all have failed due to therapeutic limitations. The search for a safe and effective drug that can reduce the many harmful effects of T2DM, including hyper-glycemia, hyperlipidemia, oxidative stress, renal damage, inflammation, and atherosclerosis, among others, is thus urgently needed. In the modern system of medicine, there is still no satisfactory effective therapy available to cure diabetes mellitus. Although insulin therapy is used for the management of diabetes mellitus there are several drawbacks to its use, which include insulin allergy, insulin antibodies, lipid dystrophy, and auto-immunity [18]. Additionally, pharmaceutical drugs such as sulfonylurea and biguanides are used for the treatment of diabetes, but these are either too expensive or have undesirable side effects or contraindications [19]. To overcome these consequences, phytochemicals and endogenous bio-molecules are being investigated for possible use in the treatment of diabetes-related complications. S-allyl cysteine (a phytochemical), sulfur-containing amino acid, is obtained from Allium Plants such as Garlic (Allium sativum, Liliaceae) [20]. S-allyl cysteine (SAC) has been reported to possess antioxidant [21] antihepatotoxic [22], neurotrophic [23], and anti-cancer activities [24].

Taurine (an endogenous biomolecule), a sulfur-containing amino acid, is present in most mammals with variation in concentration from micro to millimolar. A normal human of about 70 kg contains 1% of its total weight, as taurine *e.g.* 70 gm [25, 26]. Dietary sources of taurine (TAU) are meat and seafood, especially shellfish such as mussels, clams and oysters. Those who do not eat these foods regularly, especially vegetarians, may be at risk of TAU deficiency [27]. TAU has been reported to be hepatoprotective [28], neuroprotective [29], antioxidant [30], nephroprotective [31], anti-hypertensive [32], cardioprotective [33]. SAC and TAU have been reported to have multiple beneficial effects in combating several metabolic disorders and related complications. Despite numerous pharmacotherapeutic properties of SAC and TAU, their combined beneficial effects as an antioxidant & antidiabetic had not been explored, and scientific data is also lacking to be exposed as yet. The present study comprised an attempt to explore the *in-vitro* antioxidant and antidiabetic effects of the combined action of SAC and TAU.

MATERIALS AND METHODS:

Chemicals and Reagents: Nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), 1-chloro-2, 4-

dinitrobenzoic acid (CDNB), ethylene diamine tetraacetic acid (EDTA), and trichloroacetic acid (TCA) were purchased from Sigma–Aldrich, Chemicals Pvt. Ltd., India. Sulfosalicylic acid (SSA), bovine serum albumin (BSA), and sodium dodecyl sulfate (SDS) were purchased from Srl and Merck Chemical Pvt. Ltd. India. S-Allyl Cysteine (SAC) and Taurine (TAU) were gifted from LGC Prochem, Bangalore, India. All the other chemicals were of analytical reagent grade.

***In-vitro* Antioxidant Activity:**

Method 1: *In-vitro* Antioxidant Activity by Reducing Power Method: The reducing power of the test compound was determined according to the method described by Magdalena *et al.* (2002). Different concentrations of test compounds (10 µg ml^{-1} – 50 µg ml^{-1}) in 1 ml of distilled water were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. 2.5 ml of TCA (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and ferric chloride (0.5 ml. 0.1%), and the absorbance was measured at 700 nm (Shimadzu UV-Vis 1601). Ascorbic acid was used as a reference standard.

Method 2: *In-vitro* Antioxidant Activity by Hydrogen Peroxide Scavenging Method: The ability of test compounds to scavenge hydrogen peroxide was determined according to the method of Nagendran *et al.,* (2005). A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4), and concentration was determined spectro-photometrically at 230 nm (Shimadzu UV-Vis 1601). Standard (ascorbic acid) and the test compounds were prepared at concentrations of 5 µg/ml – 25 µg/ml in distilled water. Aliquots of standard and test compounds were added to a hydrogen peroxide solution (0.6 ml, 2 mM). The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm against a blank solution in phosphate buffer without hydrogen peroxide.

The percentage of scavenging was calculated as follows:

% H_2O_2 Scavenging = [(Absorbance of Control - Absorbance of Sample)/ Absorbance of Control] × 100

Where the absorbance of Control was the absorbance in the absence of standard or test compound

The absorbance of the Sample was the absorbance in the presence of standard or test compound

Method 3: *In-vitro* Antioxidant Activity by 2, 2-diphenyl-1-picryl Hydrazyl Radical (DPPH) Scavenging Method: The free radical scavenging capacity of the test compounds was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. The test compounds were mixed with 95% methanol to prepare the stock solution (1 mg/ml).

Freshly prepared DPPH solution was taken in test tubes, then test compound was added following serial dilutions (10 µg to 50 µg) to every test tube so that the final volume remained 3 ml. After 10 min, absorbance was read at 517 nm using a spectrophotometer (Shimadzu UV-Vis 1601, Japan). Ascorbic acid was used as a reference standard. The percent inhibition was calculated as follows [36]:

% inhibition of DPPH = [A (b) – A (s) / A (b)] × 100

Where, A (b) = absorbance of blank, and A (s) = absorbance of the sample.

***In-vitro* Antidiabetic Activity:**

Method 1: α-amylase Inhibition Study: The test compounds were weighed and serial dilutions of 10-50 µg/ml were made up with equal volumes of dimethylsulfoxide and distilled water. Then these dilutions were added to 25 µl of 20 mM phosphate buffer pH 6.9, containing porcine α- amylase at a concentration of 0.5 mg/ml and incubated at 25 ºC for 10 min. After pre-incubation, 25 µl of 0.5% starch solution in 20 mM phosphate buffer, pH 6.9, was added. The reaction mixtures were then incubated at 25 ºC for 10 min. The reaction was stopped with 50µl of 96 mM 3,5-dinitrosalicylic acid (DNS) color reagent. Absorbance was measured at 540 nm. The percent of α-amylase inhibition was calculated as follows [37]:

% inhibition = [Absorbance (control) - Absorbance (test) / Absorbance (control)] × 100

The inhibitory concentrations of the test compounds required to inhibit the activity of the

enzyme by 50% (IC_{50}) were determined from plots of percent inhibition versus log inhibitor concentration and were calculated by nonlinear regression analysis from the mean inhibitory values. Acarbose was dissolved in distilled water and serial dilutions of 10-50 µg/ml were made and used as a positive control. Experiments were performed in triplicate.

Method 2: α-glucosidase Inhibition Study: The test compounds were weighed, and serial dilutions of 10-50 µg/ml were made up with equal volumes of dimethylsulfoxide and distilled water.

Yeast α-glucosidase was dissolved at a concentration of 0.1 U/ml in 100 mM phosphate buffer pH 7.0 (used as enzyme source) containing bovine serum albumin (BSA) 2000 mg/ml and sodium azide 200 mg/ml. Paranitrophenyl- α-d-glucopyranoside was used as a substrate. 10 microliters of test compound dilutions were incubated for 5 min with a 50 µl enzyme source.

After the incubation, 50 µl of the substrate was added and further incubated for 5 min at room temp. The pre- substrate and post substrate addition absorbance were measured at 405 nm on a microplate reader. The increase in absorbance on substrate addition was obtained. The percent of α-glucosidase inhibition was calculated as follows [38]:

$$\% \text{ inhibition of } \alpha\text{-glucosidase} = [(1-B/A)] \times 100$$

Whereas A was the absorbance of control and B was the absorbance of samples containing test compounds.

The inhibitory concentrations of the test compounds required to inhibit the activity of the enzyme by 50% (IC_{50}) were determined from plots of percent inhibition versus log inhibitor concentration and were calculated by nonlinear regression analysis from the mean inhibitory values. Acarbose was dissolved in distilled water, and serial dilutions of 10-50 µg/ml were made and used as a positive control. Experiments were performed in triplicate.

RESULTS:

***In-vitro* Antioxidant Activity Analysis:**

Reducing Power Analysis of SAC, TAU, and SAC/TAU: Reducing power of SAC, TAU, and SAC/TAU was estimated by using the ascorbic acid solution as standard. The absorbance data in **Table 1** were recorded against the selected concentrations (10 - 50 µg/ml).

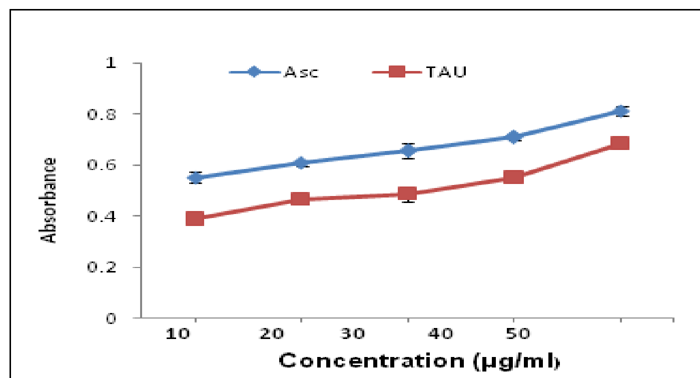
The absorbance vs. concentration curves of ascorbic acid with SAC, TAU, and SAC/TAU (Fig. no. 1) were plotted.

The Fe^{3+}–Fe^{2+} transformation was investigated in the presence of SAC, TAU, and SAC/TAU for the measurements of the reducing ability.

TABLE 1: REDUCING POWER DATA OF ASCORBIC ACID, SAC, TAU, AND SAC/TAU AT 700 NM

S. no.	Conc. (µg/ml)	Ascorbic acid (Mean±SEM)	SAC (Mean±SEM)	TAU (Mean±SEM)	SAC/ TAU (Mean±SEM)
1	10	0.552±0.03421	0.445±0.00286	0.389±0.05665	0.497±0.04543
2	20	0.608±0.05654	0.498±0.01987	0.465±0.04236	0.512±0.00623
3	30	0.656±0.01232	0.499±0.00873	0.467±0.00987	0.582±0.06523
4	40	0.698±0.06562	0.522±0.02165	0.504±0.03213	0.696±0.08754
5	50	0.812±0.02539	0.622±0.05621	0.638±0.00976	0.799±0.05028

Values represented as Mean ± S.E.M.



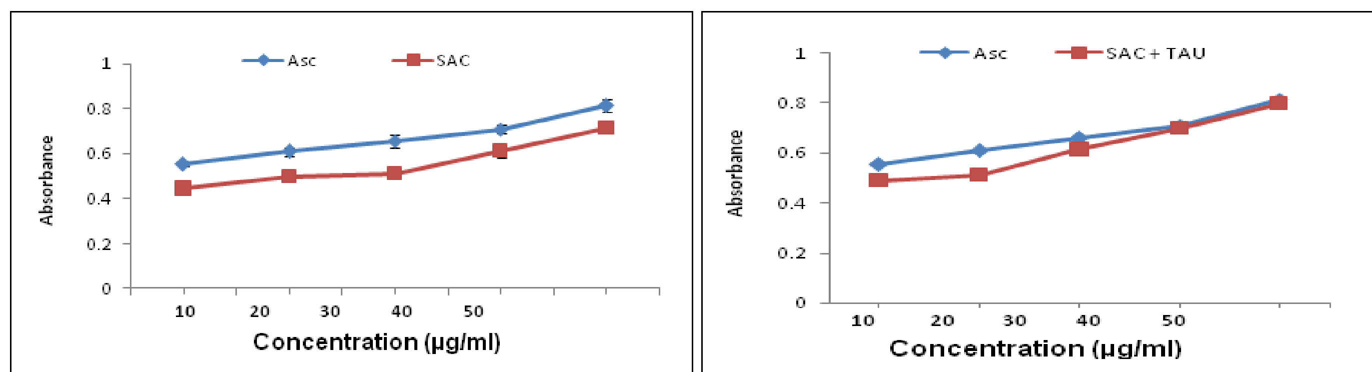


FIG. 1: REDUCING POWER CURVES OF SAC, TAU, SAC/TAU WITH ASCORBIC ACID AT 700 NM

Hydrogen Peroxide Scavenging Analysis of SAC, TAU as Individual and in Combined form (SAC/TAU): Hydrogen peroxide radical scavenging of SAC, TAU, and SAC/TAU was estimated by using the ascorbic acid solution as standard. The inhibition data in **Table 2** were recorded against the selected concentration (10 - 50 µg/ml).

Standard inhibition curves for hydrogen peroxide radical scavenging of SAC, TAU, and SAC/TAU with ascorbic acid **Fig. 2** were plotted.

From these IC_{50} values of hydrogen peroxide radical scavenging of the ascorbic acid, SAC, TAU, and SAC/TAU were calculated using the regression equation.

TABLE 2: % INHIBITION DATA OF HYDROGEN PEROXIDE ACTIVITY FOR ASCORBIC ACID, SAC, TAU, AND SAC/TAU

S. no.	Conc. (µg/ml)	Ascorbic acid (Mean±SEM)	SAC (Mean±SEM)	TAU (Mean±SEM)	SAC/ TAU (Mean±SEM)
1	10	47.32± 3.21	39.76± 3.09	33.33± 3.31	45.04± 2.37
2	20	52.12± 2.31	47.32± 2.21	40.63± 2.06	50.06± 3.92
3	30	58.02± 2.11	54.06± 3.41	45.99± 2.70	56.88± 2.20
4	40	62.96± 3.82	57.95± 2.98	53.01± 3.03	60.32± 3.18
5	50	65.56± 2.24	63.55± 3.72	58.22± 3.30	63.34± 2.94

Values represented as Mean ± S.E.M.

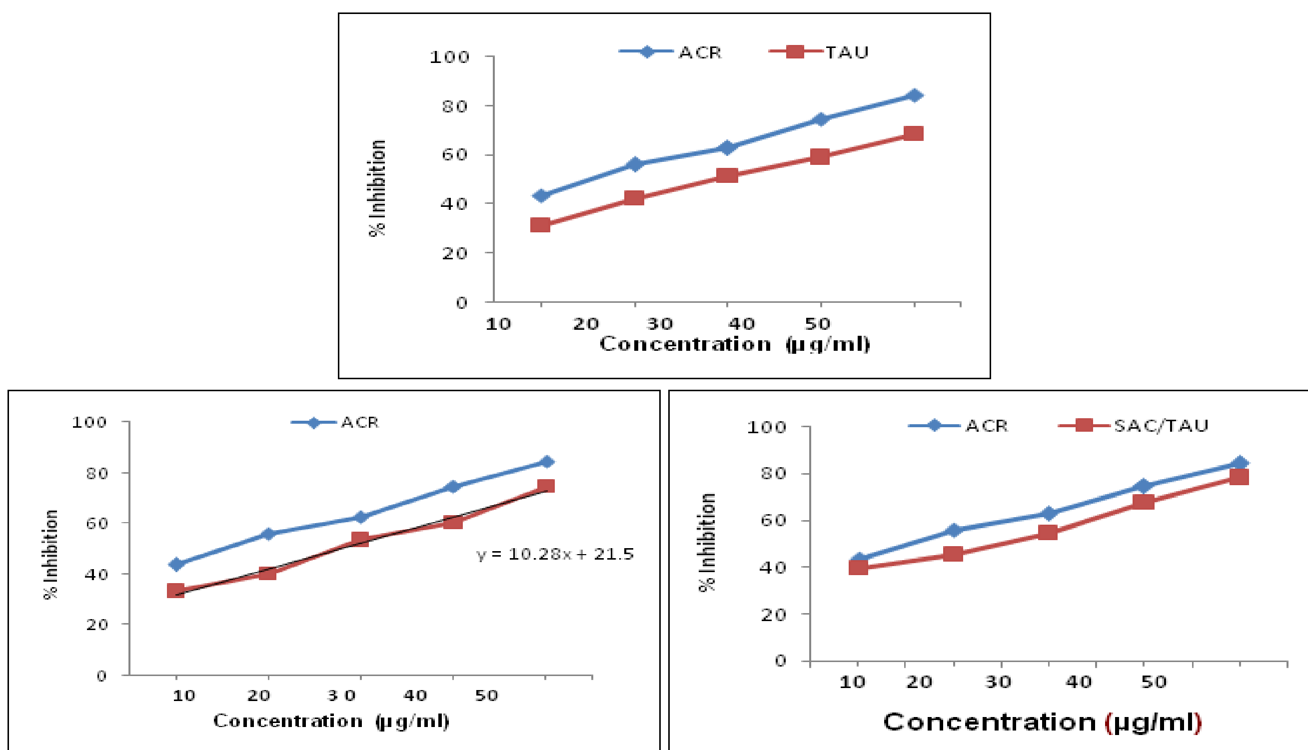


FIG. 2: H_2O_2 SCAVENGING ACTIVITY OF SAC, TAU, AND SAC/TAU WITH ASCORBIC ACID

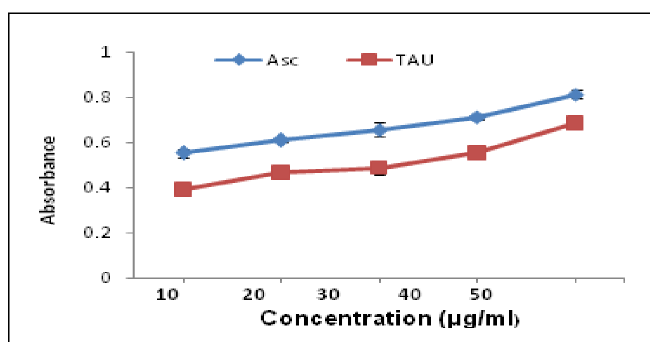
DPPH Radical Scavenging Analysis of SAC, TAU as Individual and in Combined Form (SAC/TAU): DPPH (2, 2-diphenyl-1-picryl-hydrazyl) radical scavenging of SAC, TAU, and SAC/TAU was estimated by using the ascorbic acid solution as standard. The inhibition data **Table 3** were recorded against the selected concentrations (10 - 50 µg/ml). The % inhibition curves for DPPH radical scavenging of SAC, TAU, and SAC/TAU with ascorbic acid **Fig. 3** were plotted.

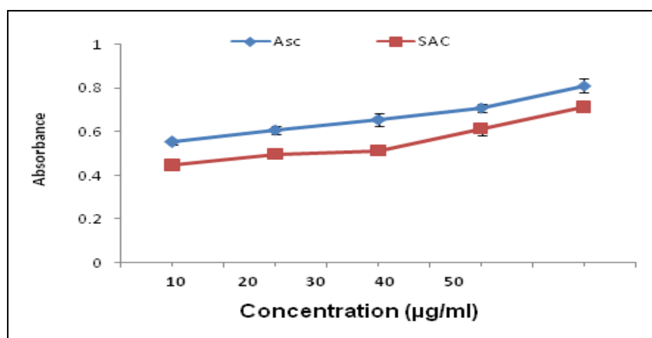
From which IC_{50} values of percentage inhibition of DPPH by ascorbic acid, SAC, TAU, and SAC/TAU were calculated using the regression equation.

TABLE 3: % INHIBITION DATA FOR DPPH ASSAY OF ASCORBIC ACID, SAC, TAU, AND SAC/TAU

S. no.	Conc. (µg/ml)	Ascorbic acid (Mean±SEM)	SAC (Mean±SEM)	TAU (Mean±SEM)	SAC/ TAU (Mean±SEM)
1	10	53.45± 1.14	45.53± 1.44	41.42± 0.35	46.76± 0.65
2	20	62.43± 0.75	52.98± 0.55	48.79± 1.54	56.65± 0.91
3	30	69.92± 2.84	58.52± 0.95	56.63± 1.74	59.43± 1.82
4	40	76.43± 0.25	68.97± 1.74	59.63± 0.95	73.74± 0.94
5	50	82.45± 0.35	72.53± 0.75	62.82± 0.05	78.32± 1.83

Values represented as Mean ± S.E.M.





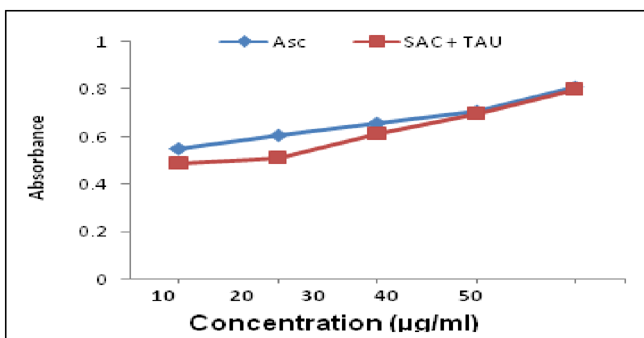


FIG. 3: DPPH RADICAL SCAVENGING ACTIVITY OF SAC, TAU, AND SAC/TAU WITH ASCORBIC ACID

***In-vitro* Antidiabetic Analysis by α-amylase Inhibitory Activity:** *In-vitro* antidiabetic activity of SAC, TAU, and SAC/TAU by α-amylase was estimated by using acarbose solution as standard. The inhibition data in **Table 4** were recorded against the selected concentrations (10 - 50 µg/ml). The % inhibition curves for α-amylase enzyme inhibition of SAC, TAU, and SAC/TAU with acarbose **Fig. 4** were plotted. The IC_{50} values of percentage inhibition of an α-amylase enzyme by acarbose, SAC, TAU, and SAC/TAU were calculated using the regression equation.

TABLE 4: α-AMYLASE INHIBITORY ACTIVITY OF ACARBOSE, SAC, TAU, AND SAC/TAU

S. No.	Conc. (µg/ml)	Acarbose (Mean±SEM)	SAC (Mean±SEM)	TAU (Mean±SEM)	SAC/ TAU (Mean±SEM)
1	10	53.45± 1.41	30.03± 0.82	21.71± 0.84	41.51± 1.03
2	20	62.43± 0.81	44.30± 1.68	37.52± 1.38	51.35± 0.98
3	30	68.92± 1.41	59.70± 0.57	45.70± 0.85	62.65± 1.27
4	40	76.43± 1.28	73.05± 1.78	57.53± 1.35	73.72± 1.18
5	50	82.45± 0.23	78.43± 0.53	69.60± 1.20	80.32± 0.71

Values represented as Mean ± S.E.M

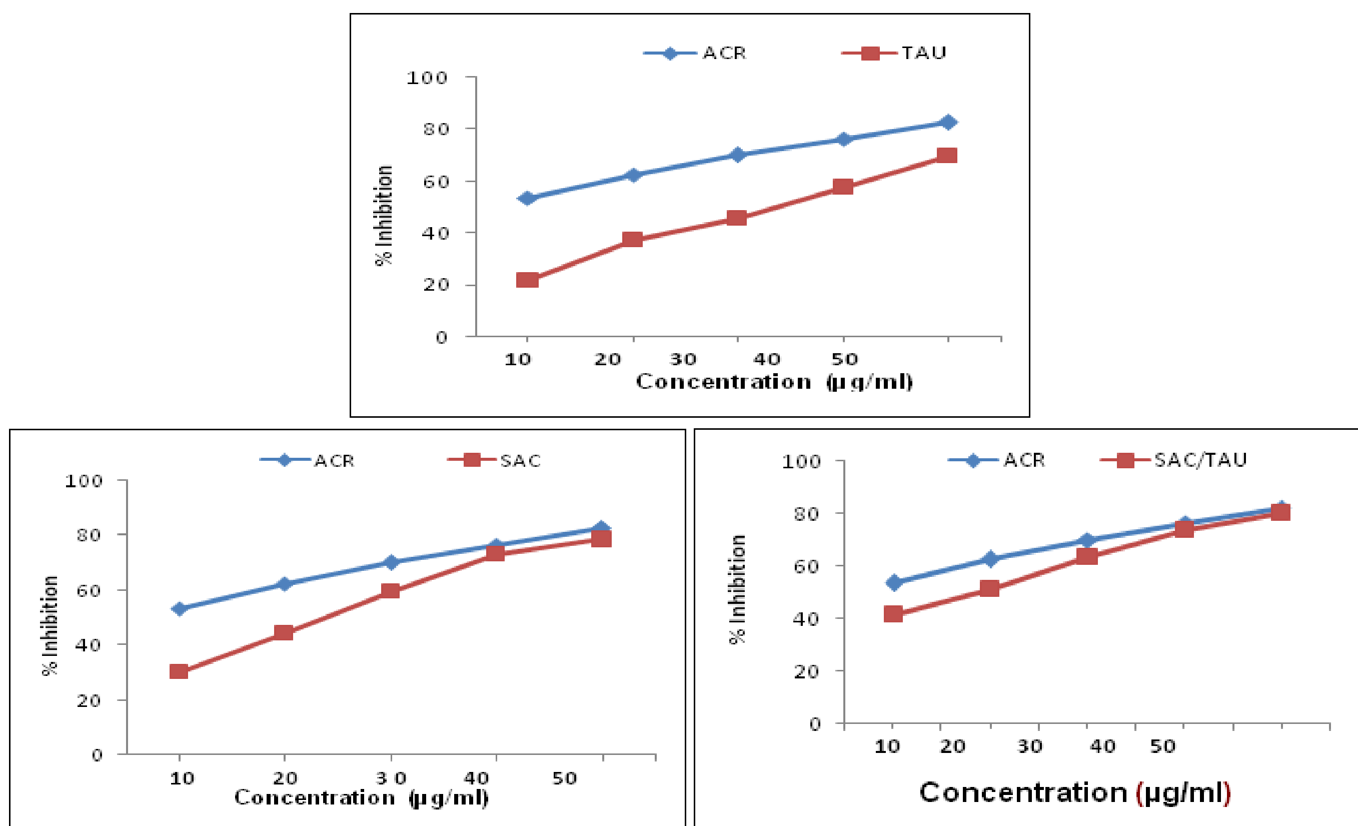


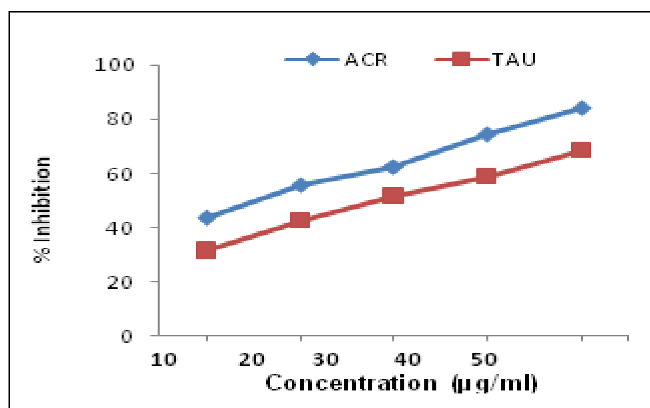
FIG. 4: α-AMYLASE INHIBITORY ACTIVITY OF SAC, TAU, AND SAC/TAU WITH ACARBOSE

***In-vitro* Antidiabetic Analysis by α-glucosidase Inhibitory Activity:** *In-vitro* antidiabetic activity of SAC, TAU, and SAC/TAU by α-glucosidase was estimated by using acarbose solution as standard. The inhibition data in **Table 5** were recorded against the selected concentrations (10 - 50 µg/ml). The % inhibition curves for α-glucosidase enzyme inhibition of SAC, TAU, and SAC/TAU with acarbose **Fig. 5** were plotted. IC_{50} values of percentage inhibition of the α-glucosidase enzyme by acarbose, SAC, TAU, and SAC/TAU were calculated using the regression equation.

TABLE 5: α- GLUCOSIDASE INHIBITORY ACTIVITY OF ACARBOSE, SAC, TAU, AND SAC/TAU

S. no.	Conc. (µg/ml)	Acarbose (Mean±SEM)	SAC (Mean±SEM)	TAU (Mean±SEM)	SAC/ TAU (Mean±SEM)
1	10	42.60± 0.25	33.21± 0.76	31.32± 0.12	39.52± 0.22
2	20	51.76± 1.98	40.21± 1.11	41.43± 1.89	43.43± 2.01
3	30	62.65± 0.12	52.54± 0.82	46.54± 2.45	58.52± 1.34
4	40	73.43± 1.26	60.21± 0.21	58.03± 0.95	63.54± 0.82
5	50	82.32± 2.44	74.65± 1.87	66.64± 2.31	75.43± 0.32

Values represented as Mean ± S.E.M



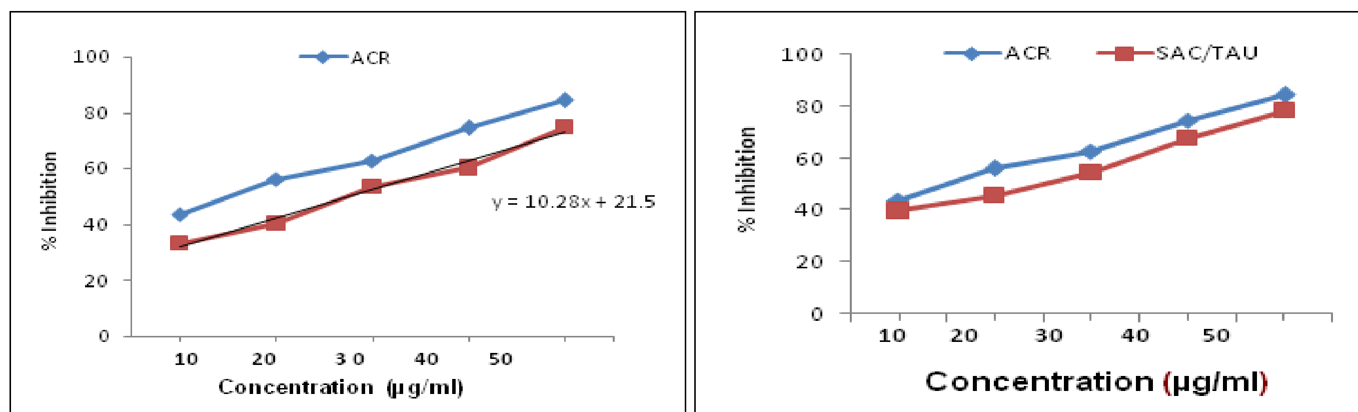


FIG. 5: α-GLUCOSIDASE INHIBITORY ACTIVITY OF SAC, TAU AND SAC/TAU WITH ACARBOSE

DISCUSSION: The reducing power of the compound may serve as a significant indicator of its potential antioxidant activity. Reducing power assay is used to evaluate the ability of natural antioxidants to donate electrons [39]. The reducing power of SAC, TAU, and SAC/TAU was found very potent, and the power of the test compounds increased with increasing dose. The result showed that SAC/TAU could reduce most of the Fe3+ ions, being more potent than SAC and TAU alone.

Hydroxy radicals (H_2O_2) accumulation can lead to a reduction in cleavage of bonds between oxygen atoms leading to the production of hydroxyl radicals, which is a very reactive and unstable oxidizing species that reacts instantaneously with any biological molecule. H_2O_2 can penetrate all biological membranes and can therefore cause cellular damage [40]. The study conclusively demonstrated hydrogen peroxide decomposition activity of ascorbic acid, SAC, TAU, and SAC/TAU in a concentration-dependent manner with an IC_{50} of 2.24, 12.61, 16.59, and 4.84 µg ml-1, respectively. The IC50 value of the SAC/TAU was found to be comparable to reference standard ascorbic acid. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between the antioxidant molecule and radical progress, results in the scavenging of the radical by hydrogen donation [41]. It is visually noticeable as discoloration from purple to yellow. Figure 5 indicates a noticeable effect of samples and fractions on the scavenging of free radicals. The study conclusively depicted that DPPH radical scavenging assay for ascorbic acid, SAC, TAU, and SAC/TAU exhibited a significant dose-dependent inhibition with IC_{50} of 6.5, 23.75, 30.10 and 16.30 µg ml-1 respectively. Inhibition of some ∝-glycosidase enzymes such as α-glucosidase and α-amylase enzymes involved in the digestion of carbohydrates can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet. It therefore can be an important strategy in the management of postprandial blood glucose level in type 2 diabetic patients and borderline patients [42]. α-amylase is responsible for the breakdown of oligo and/or disaccharide to monosaccharides.

Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a marked decrease in the rate of glucose absorption, thereby blunting the postprandial plasma glucose rise [43].

The study conclusively depicted that IC_{50} values for acarbose, SAC, TAU, and SAC/TAU were 3.7, 24.03, 33.10 and 17.26 µg/ml, respectively. The SAC and TAU produced a weak α-amylase enzyme inhibition in comparison to the combination (SAC/TAU). On the other hand, α-glucosidase enzymes in the intestinal lumen and the brush border membrane play main roles in carbohydrate digestion to degrade starch and oligosaccharides to monosaccharides before they can be absorbed.

It was proposed that suppression of the activity of such digestive enzymes would delay the degradation of starch and oligosaccharides, which would, in turn, cause a decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation [44]. The study conclusively depicted that IC_{50} values for acarbose, SAC, TAU, and SAC/TAU were 2.9, 15.9, 19.01 and 5.73 µg/ml, respectively. The SAC and TAU produced a weak α- glucosidase enzyme

inhibition in comparison to the combination (SAC/TAU).

CONCLUSION: Our *in-vitro* studies indicated that combined SAC/TAU had α-amylase and α-glucosidase inhibitory activity; thus, the combination might possess therapeutic antidiabetic effects in type 2 diabetes mellitus. The results obtained from the present *in-vitro* study will be confirmed by taking up *in-vivo* studies in the future. The basic concepts of this investigation left behind several unfolded directions for future researchers to carry out such investigations establishing novel pharmacological aspects to elucidate the synergistic actions of different combinations concerning their respective mechanisms of actions.

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AVAILABILITY OF DATA AND MATERIAL: All data generated during this study are included in this published article.

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CONFLICTS OF INTEREST: The authors declare that there are no conflicts of interest.

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