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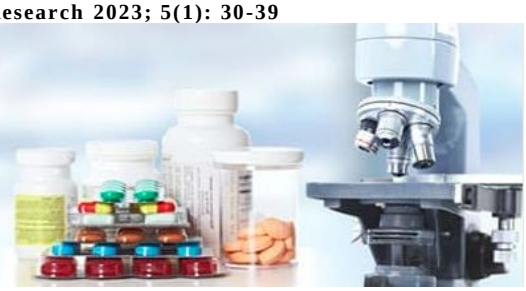

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In vitro anti-oxidant and anti-diabetic activity of Brucine and Eugenol in combination

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Abstract
The present study evaluates the *In vitro* anti-oxidant and *In vitro* anti-diabetic activity of Brucine and eugenol in combination in various models. The anti-oxidant activity by hydrogen peroxide method was performed it has IC_{50} value of 39.52 µg/ml. The Ferrous reducing activity of Brucine and eugenol in combination has IC_{50} value 16.33 µg/ml. The phytochemical in combination has shown α-Amylase inhibitory activity and has IC_{50} value of 55.83 µg/ml. The *In vitro* studies clearly indicate that Brucine and eugenol in combination has synergic effect against oxidation reaction and in the treatment of diabetes when compared to individual therapy with Brucine and eugenol alone.

Keywords: Brucine, eugenol, *in vitro* anti-oxidant activity, *in vitro* anti-diabetic activity, hydrogen peroxide method, FRAP Method, α- amylase method

Introduction

Diabetes mellitus

Diabetes mellitus is a chronic disease which is characterized by raised blood glucose levels which alters the body metabolism through fat and protein metabolic changes. The higher levels of blood glucose levels may be caused due to (a) lack of insulin secretion (b) Inability of cells to effectively use the insulin which is being released [1]. It is classified in to (a) Type 1 diabetes (b) Type 2 diabetes (c) Gestational diabetes [2]. Diabetes is an important pathological condition which can cause chronic ill health and premature mortality [3].

Insulin is an endocrine peptide hormone which binds to receptors of plasma membrane-bound in target cell [4]. Insulin is secreted from pancreatic beta cells, which undergo liver degradation and reaches systemic circulation and cleared by kidneys. The insulin and glucose systems coincide by feedback control signals [5].

Pathogenesis of diabetes is a complex mechanism, where numerous factors are involved. It may be due to family history of type 1 diabetes, genetic factors, Life style modifications which leads to change in increased Body mass index (BMI), due to physical inactivity, poor nutrition, hypertension, smoking [6]. Stress is also a major contributor to chronic hyperglycemia in diabetes [7].

Hyperglycemia can cause the activation of various cellular pathways, which includes increased oxidant stress, increased flux in to the polyol and hexosamine pathway, PKC activation, and transforming growth factor (TGF)-β-SMAD-MAPK Signaling pathways all these pathways can damage kidneys and even lead to multiple diseases [8].

Role of alkaloids in the treatment of diabetes.

Alkaloids are naturally derived chemical compounds which are basic due to presence of at least one nitrogen atom in their heterocyclic ring. They have wide range of biological activities in treating various diseases. In recent years alkaloids play an important role in the treatment of diabetes and its related complications [9].

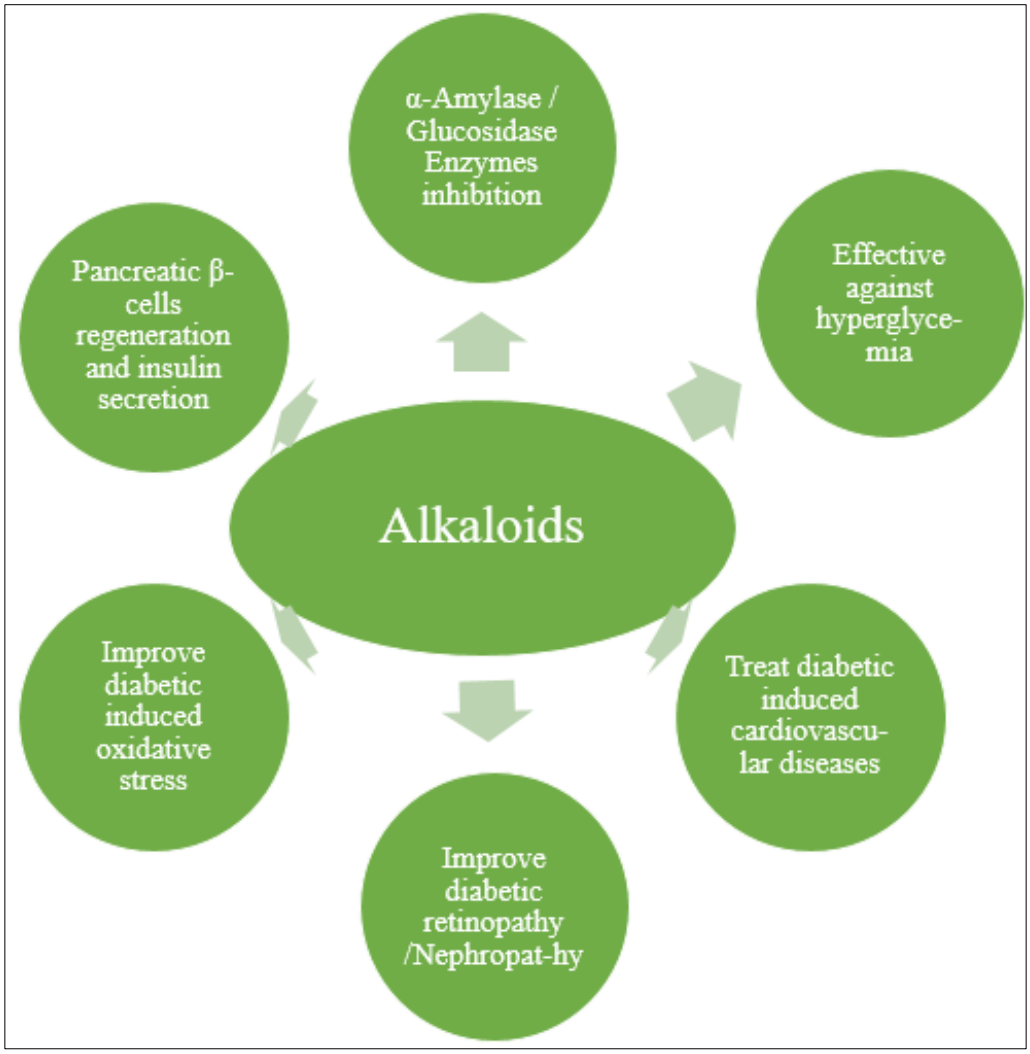


Fig 1: Role of alkaloids in the treatment of diabetes [9]

Brucine: Brucine is an alkaloid, it consists of indole ring which makes it weak alkaline indole alkaloid [10]. Brucine has been used in treating various diseases like diabetes, gonorrhea, bronchitis. It is also used for liver cancer and rheumatic pain as it improves blood circulation according to Chinese medicine system [11]. It can also act as analgesic, anti-inflammatory, anti-oxidant and anti-snake venom [12, 13]. It has narrowed applications in the treatment of malignant tumors due to its high toxicity and narrow therapeutic window [14].

Physicochemical Properties

Brucine is a weak Indole alkaloid, basic in nature. Its chemical formula is $C_{23}H_{26}N_2O_4$, molecular weight is 394.47 Da. Physical properties are it is a white crystal that is highly toxic and odorless, with bitter taste. It is slightly soluble in water and soluble in ether, chloroform, ethanol, methanol and other organic solvents. It has high toxicity, poor water solubility and short half-life [15].

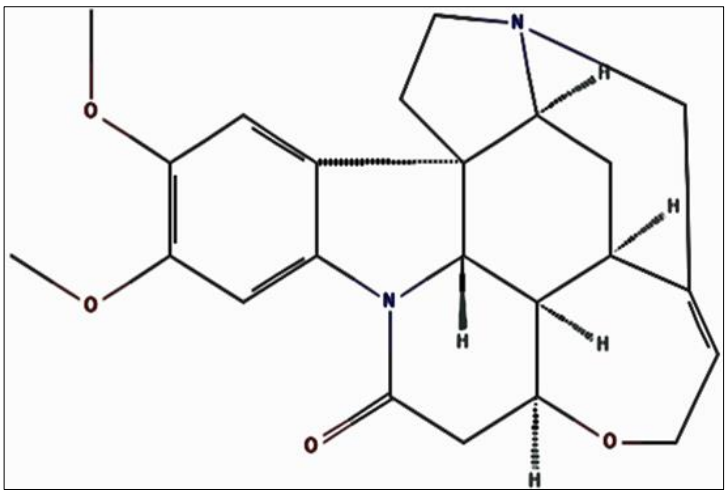


Fig 2: Chemical structure of brucine

Role of Phenylpropanoids in the treatment of diabetes. Various *in vivo* and *in vitro* models have been explored to check the anti-diabetic effects of Phenylpropanoids in streptozotocin induced rats. The following observations were recorded.

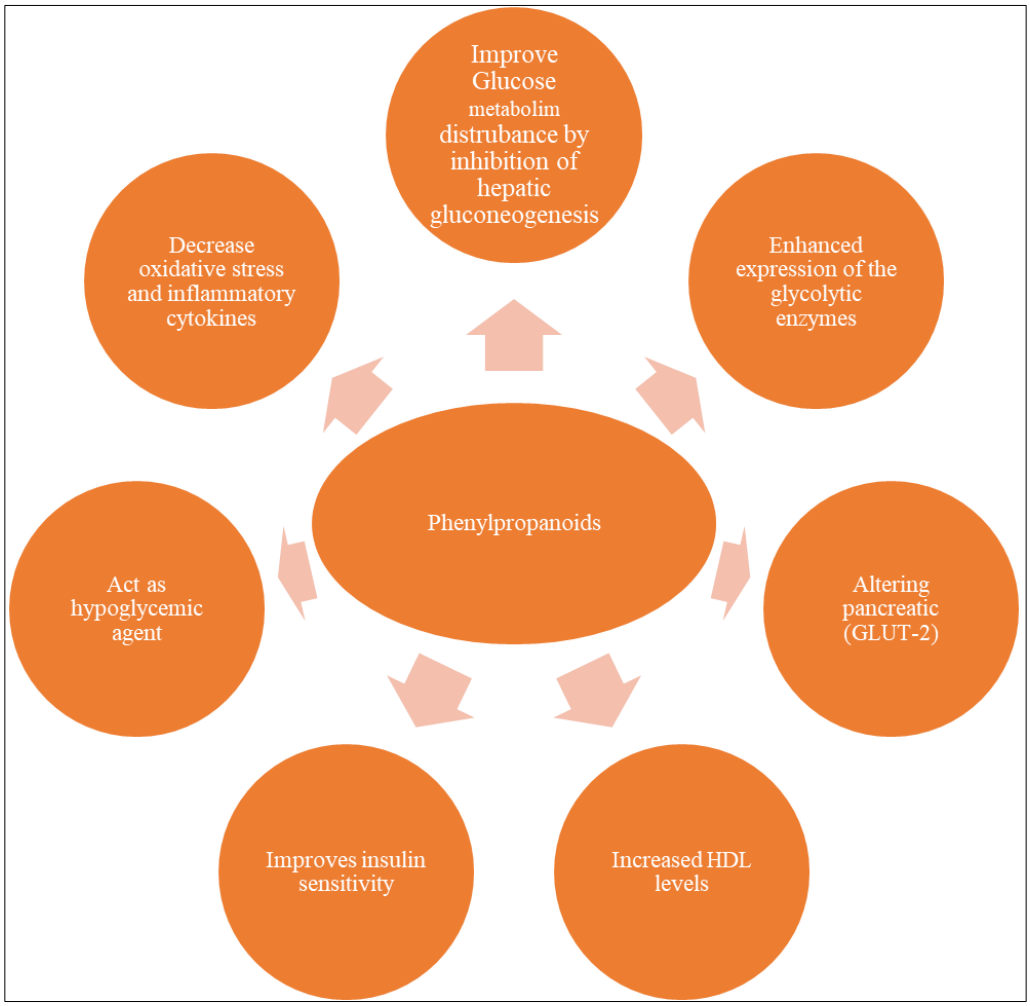


Fig 3: Phenylpropanoids in the treatment of diabetes.

Eugenol

Eugenol is a phenylpropanoid, it is a liquid with pale yellow color [16]. Eugenol shows antioxidant activity, anti-inflammatory action, antibacterial, antiviral effects [17], analgesic activity, protective effect, anesthetic action, anticonvulsant activity, cardiovascular actions, anticancer activity, antigenotoxic effect [18], anti-diabetic effect [19].

Physicochemical Properties

Eugenol molecular weight is 164.2011g/mol and chemical formula is $C_{10}H_{12}0_2$, it is a clear colorless or pale-yellow liquid. It has strong aromatic odor same as clove, and has pungent spicy taste. It darkens and thickens when exposed to air. It is soluble in volatile oils, glacial acetic acid, alkalis, methanol, and insoluble in water [20].

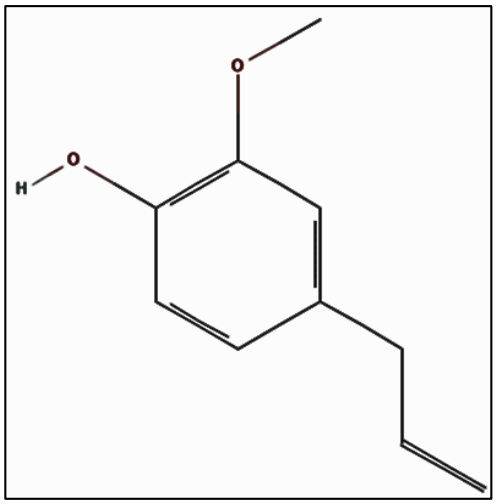


Fig 4: Chemical structure of Eugenol

Anti-Oxidants

Oxygen free radicals (OFR) are continuously generated in cells exposed to an aerobic environment [21]. Reactive oxygen species (ROS) including all other free radicals such as superoxide radical (O^{2-}), hydroxyl radical (OH^{-}), singlet oxygen (O_2) and non-free radical species such as hydrogen peroxide (H_2O_2) are various forms of oxygen and are generated by biological interactions or exogenous factors as an oxidation product [22, 23].

Materials and Methods

***In Vitro* Anti-Oxidant Activity**

Scavenging of hydrogen peroxide

Chemicals used

Hydrogen peroxide, Ascorbic acid, phosphate buffer.

Procedure

The ability of both Brucine and eugenol together to scavenge hydrogen peroxide was determined by method of Saumya and Basha [24]. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer (pH 7.4). Ascorbic acid (1-5 µg/ml) was used as reference standard. Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm. The sample solution was prepared by 1:1 ratio of brucine and eugenol dissolved in methanol at concentration of (10-50 µg/ml). 1 ml of sample solution was added to 2 ml of hydrogen peroxide solution. The above Solution was kept aside for 10 mins. Blank solution was prepared by adding phosphate buffer without adding hydrogen peroxide. Absorbance of hydrogen peroxide was determined at 230 nm. The percentage of scavenging of hydrogen peroxide of both Sample and standard compounds.

$$\%\ scavenged\ H2O2=\frac{(A\ control-(Asample-A\ sample\ blank))}{A\ control}\times 100$$

A control indicates the absorbance of control containing 2 ml of H_2O_2 and 1 ml of phosphate buffer. A Sample is the absorbance of sample. A Sample Blank is the absorbance of sample blank containing 2 ml of phosphate buffer and 1 ml of sample solution dissolved in methanol. Phosphate buffer is used as blank.

The IC_{50} H_2O_2 values (the concentration of sample required for inhibition of 50% of H_2O_2 molecules) were obtained from the linear regression line. The antioxidant activity was evaluated based on this IC_{50} value [25].

Ferrous Reducing antioxidant power (FRAP) assay

Chemicals used

Sodium acetate buffer, 2,4,6-Tripyridyl-S-triazine (TPTZ), HCl, $FeCl_3$.

Procedure

The reducing power of brucine and eugenol was determined by Benzie and Strain method [26]. FRAP reagent was a mixture (10:1:1. v/v/v) of 300mM sodium acetate buffer (pH 3.6), 10 mM2, 4, 6-Tripyridyl-S-triazine (TPTZ) in 40 mM HCl and $FeCl_3$. $6H_2O$ (20mM). A calibration curve was plotted with different concentration of ferrous sulphate $FeSO_4$ (0.2-1.0 mM) as the standard. Ascorbic acid (1-5 µg/ml) was used as reference standard. Different amounts of sample solution were prepared by 1:1 ratio of brucine and eugenol dissolved in methanol at concentration of (5-25 µg/ml). 1 ml of sample solution was mixed with 3 ml of freshly prepared FRAP reagent. The mixture was incubated at 37 °C for 30 min. The absorbance was measured at 593 nm. The anti-oxidant capacity based on the ability to reduce ferric ions of sample was determined using linear regression equation obtained from calibration curve of $FeSO_4$ and expressed as mM $FeSO_4$ equivalent per gram of sample [25].

$$\%\ Reducing\ power=\frac{A0-A1}{A0}\times 100$$

Where A0 was the absorbance of the control, and A1 was the absorbance in the presence of the sample [27].

***In vitro* Anti- Diabetic Activity**

Inhibition of alpha-amylase enzyme

Chemicals used

Alpha amylase, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, HCl, NaOH, starch, DNSA color reagent, sodium potassium tartrate tetrahydrate, Acarbose.

Buffer preparation

Prepare 800 ml of distilled water in a container, add 2.861 g of sodium phosphate dibasic heptahydrate and 1.287g sodium phosphate monobasic monohydrate to the solution. Adjust the pH of the solution to 6.9 using either NaOH or HCl and make up the volume to 1 L.

Starch solution

Take motor and pestle and add 500 mg of starch to it and add few drops of distilled water to make a thin paste, transfer the paste to 50 ml beaker. Take 250 ml beaker and add 100 ml distilled water to it and heat the water to boiling. Slowly pour the paste in to boiling water and keep stirring constantly using glass rod. Boil it for 10 mins, transfer to beaker, cool it.

DNSA color reagent preparation

Dissolve 0.1 g of 3, 5 dinitro salicylic acid in 5 ml water. Add slowly 3 gm of sodium potassium tartrate tetrahydrate. Add 2 ml of 2N NaOH (8 g of NaOH in 100 ml water). Dilute to final volume 10 ml with water.

Procedure

500 µl (0.5ml) of test samples brucine and eugenol are taken in 1:1 ratio and dissolved in methanol at different concentration (20-100 µg/ml) was mixed with 500 µl of α-amylase (0.5mg/ml) solution with 0.20 mM phosphate buffer (pH 6.9). This mixture was incubated at 25 °C for 10 min and 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The resultant mixtures were then incubated at 25 °C for 10 min. This reaction was terminated using 1.0 ml of 3, 5 dinitro salicylic acid color reagent. At this time, the test tubes were placed in a boiling water bath (100 °C) for 5 min, cooled until room temperature was attained. The mixture was then diluted by adding 10 ml distilled water and absorbance was measured at 540 nm. The absorbance of blank (Buffer instead of sample and Amylase solution). Control (Buffer instead of sample). Acarbose was used as standard drug (10-100µg/ml). The inhibition of α-amylase was calculated using the formula. Calculation of 50% Inhibitory

Concentration (IC_{50}): The concentration of sample required to scavenge 50% of the radicals IC_{50}.

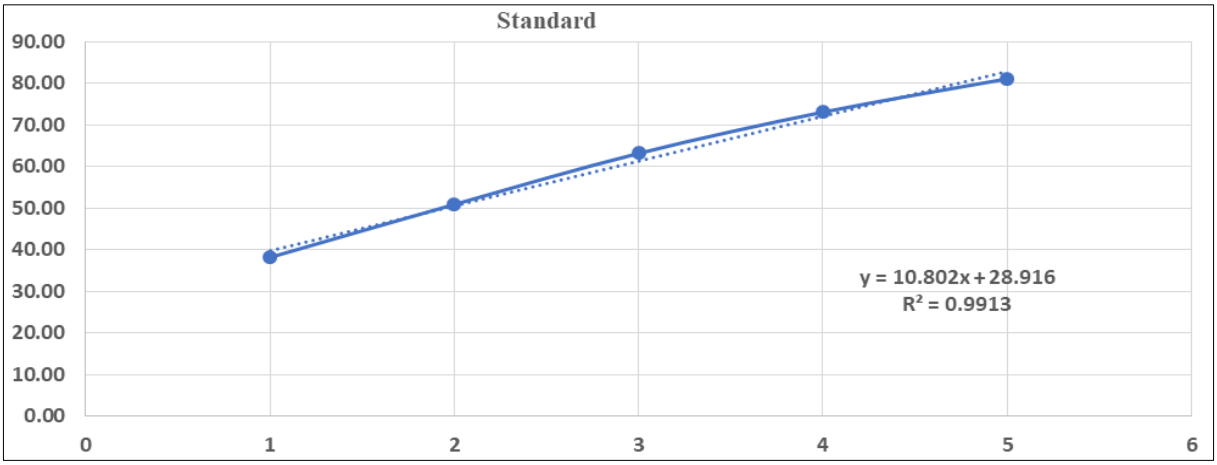
$$\%\ Inhibition\ of\ \alpha - Amylase\ = \frac{A0 - A1}{A0} \times 100$$

Where, A0 is absorbance of control and A1 is absorbance of sample [28].

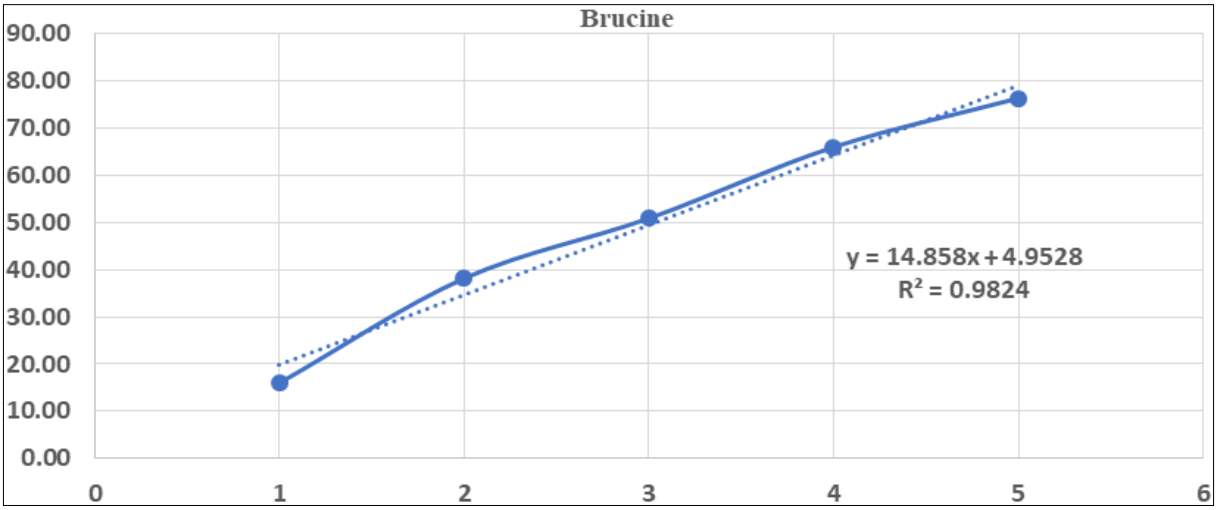
Results

Table 1: Anti-oxidant Activity of Ascorbic acid, Brucine, Eugenol and Brucine+ Eugenol by Hydrogen Peroxide Method

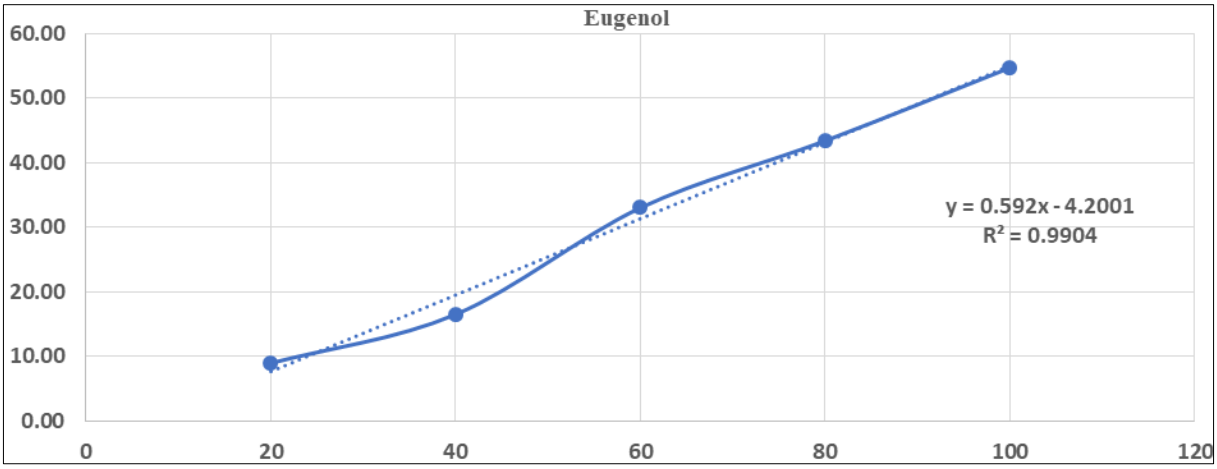
Drug	Concentration (µg /ml)	Sample	Blank	Sample - Blank	Control	(Control Abs)-(Sample - Blank)	% Inhibition	IC_{50} (µg/ml)
Standard	1	0.133	0.002	0.131	0.212	0.081	38.21	1.95±0.05
	2	0.106	0.002	0.104	0.212	0.108	50.94	
	3	0.08	0.002	0.078	0.212	0.134	63.21	
	4	0.059	0.002	0.057	0.212	0.155	73.11	
	5	0.042	0.002	0.040	0.212	0.172	81.13	
Brucine	1	0.180	0.002	0.178	0.212	0.034	16.04	3.03±0.03
	2	0.133	0.002	0.131	0.212	0.081	38.21	
	3	0.106	0.002	0.104	0.212	0.108	50.94	
	4	0.074	0.002	0.072	0.212	0.14	66.04	
	5	0.052	0.002	0.050	0.212	0.162	76.42	
Eugenol	20	0.195	0.002	0.193	0.212	0.019	8.96	91.55±0.97
	40	0.179	0.002	0.177	0.212	0.035	16.51	
	60	0.144	0.002	0.142	0.212	0.07	33.02	
	80	0.122	0.002	0.12	0.212	0.092	43.40	
	100	0.098	0.002	0.096	0.212	0.116	54.72	
Brucine + Eugenol	10	0.182	0.002	0.18	0.212	0.032	15.09	39.52±0.79
	20	0.162	0.002	0.16	0.212	0.052	24.53	
	30	0.123	0.002	0.121	0.212	0.091	42.92	
	40	0.108	0.002	0.106	0.212	0.106	50.00	
	50	0.084	0.002	0.082	0.212	0.13	61.32	



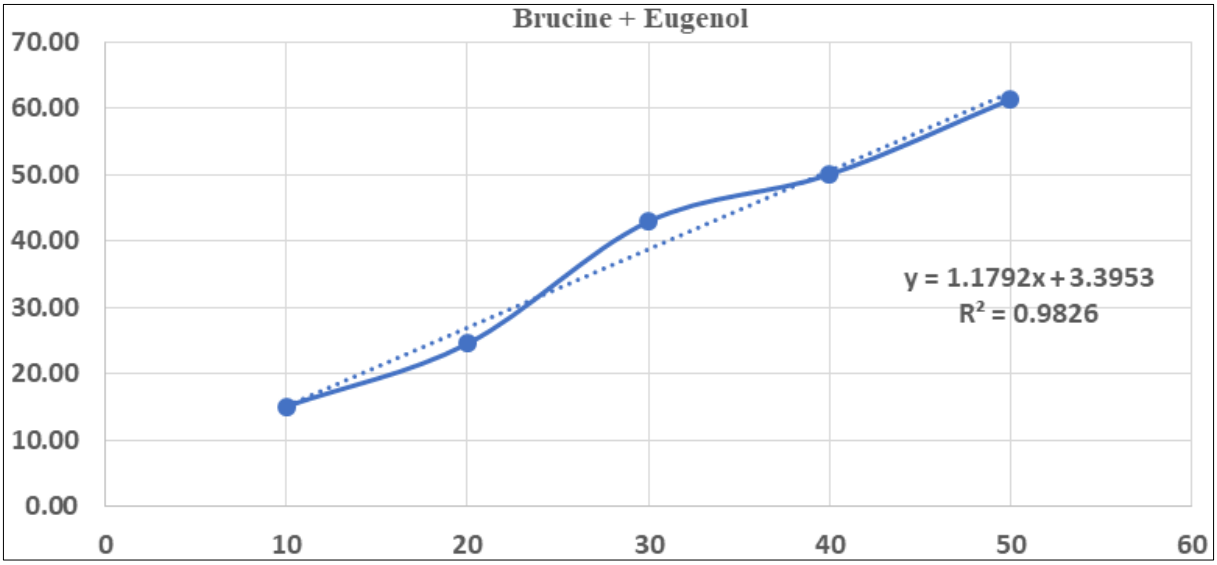
Graph 1: Ic_{50} value of standard by H2O2 method



Graph 2: Ic_{50} value of Brucine by H2O2 method



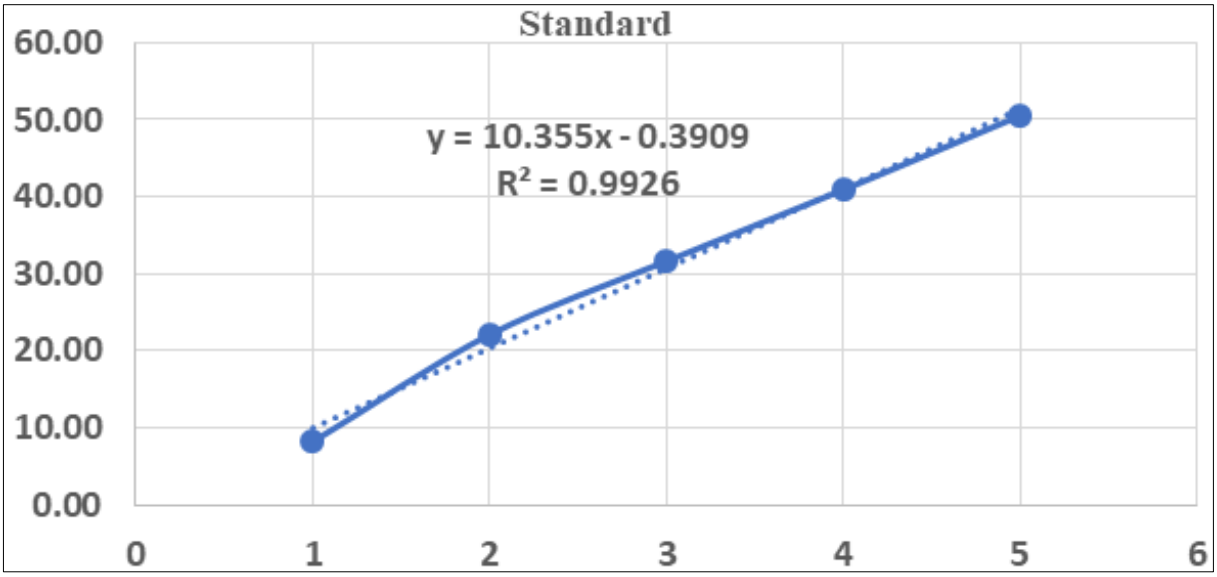
Graph 3: Ic_{50} value of Eugenol by H2O2 method



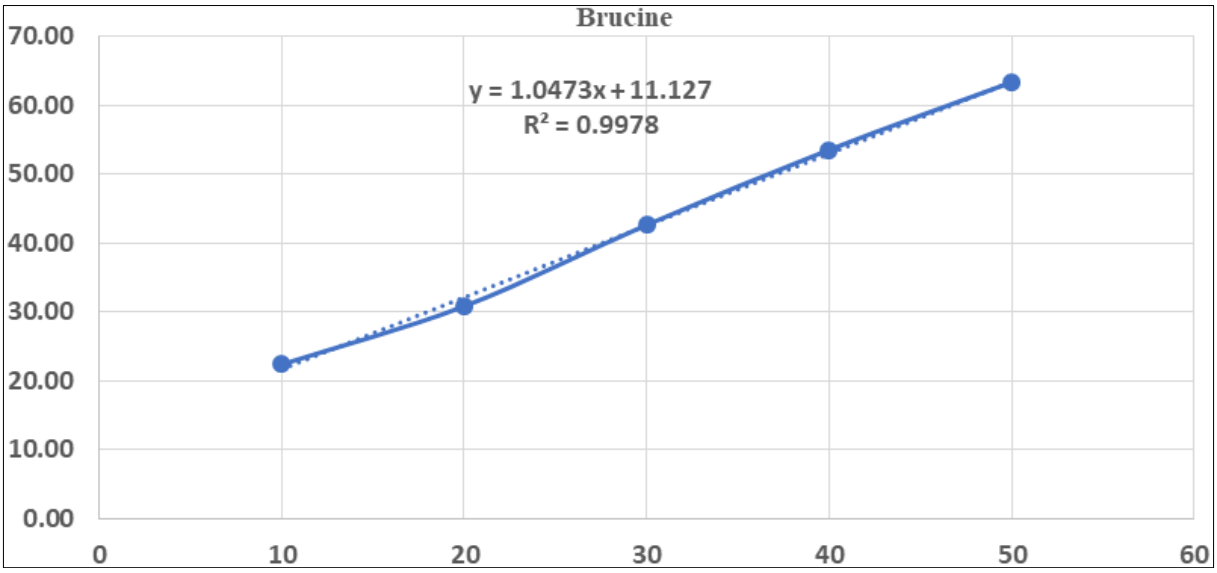
Graph 4: Ic_{50} value of Brucine +Eugenol by H2O2 method

Table 2: Anti-oxidant Activity of standard, Brucine, Eugenol and Brucine+ Eugenol by FRAP Method

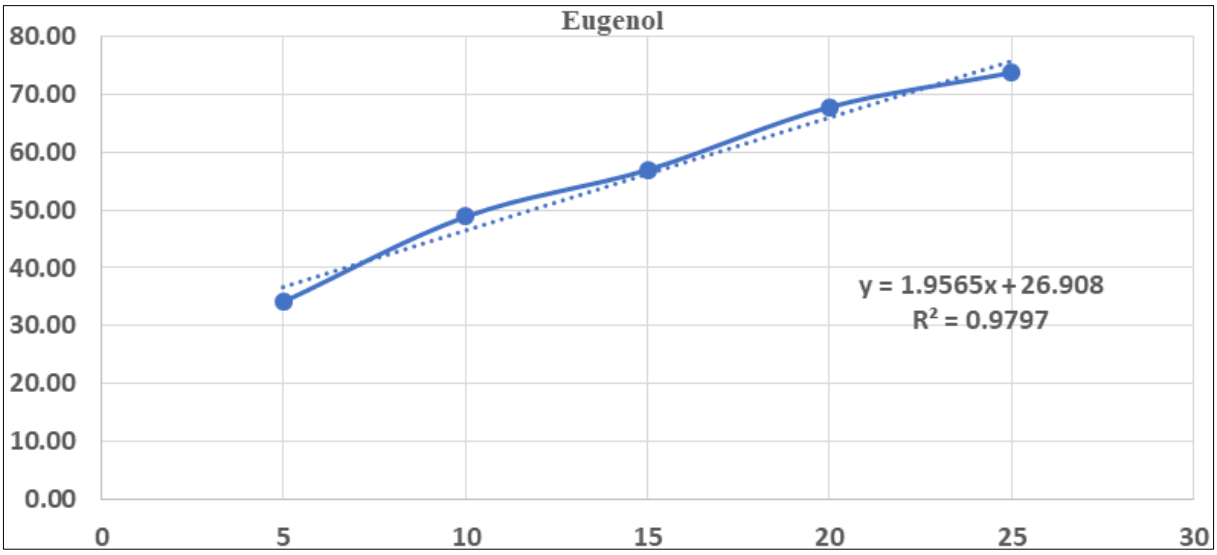
Drug	Concentration (µg /ml)	Sample	Blank	Sample - Blank	Control	(Control Abs)-(Sample Blank	% Inhibition	IC_{50}(µg/ml)
Standard	1	1.011	0.001	1.01	1.1	0.09	8.18	4.86±0.43
	2	0.858	0.001	0.857	1.1	0.243	22.09	
	3	0.753	0.001	0.752	1.1	0.348	31.64	
	4	0.651	0.001	0.65	1.1	0.45	40.91	
	5	0.545	0.001	0.544	1.1	0.556	50.55	
Brucine	10	0.855	0.001	0.854	1.1	0.246	22.36	37.11±1.3
	20	0.762	0.001	0.761	1.1	0.339	30.82	
	30	0.632	0.001	0.631	1.1	0.469	42.64	
	40	0.512	0.001	0.511	1.1	0.589	53.55	
	50	0.404	0.001	0.403	1.1	0.697	63.36	
Eugenol	5	0.725	0.001	0.724	1.1	0.376	34.18	11.80±0.43
	10	0.564	0.001	0.563	1.1	0.537	48.82	
	15	0.475	0.001	0.474	1.1	0.626	56.91	
	20	0.356	0.001	0.355	1.1	0.745	67.73	
	25	0.291	0.001	0.29	1.1	0.81	73.64	
Brucine + Eugenol	5	0.818	0.001	0.817	1.1	0.283	25.73	16.33±0.56
	10	0.701	0.001	0.7	1.1	0.4	36.36	
	15	0.586	0.001	0.585	1.1	0.515	46.82	
	20	0.449	0.001	0.448	1.1	0.652	59.27	
	25	0.358	0.001	0.357	1.1	0.743	67.55	



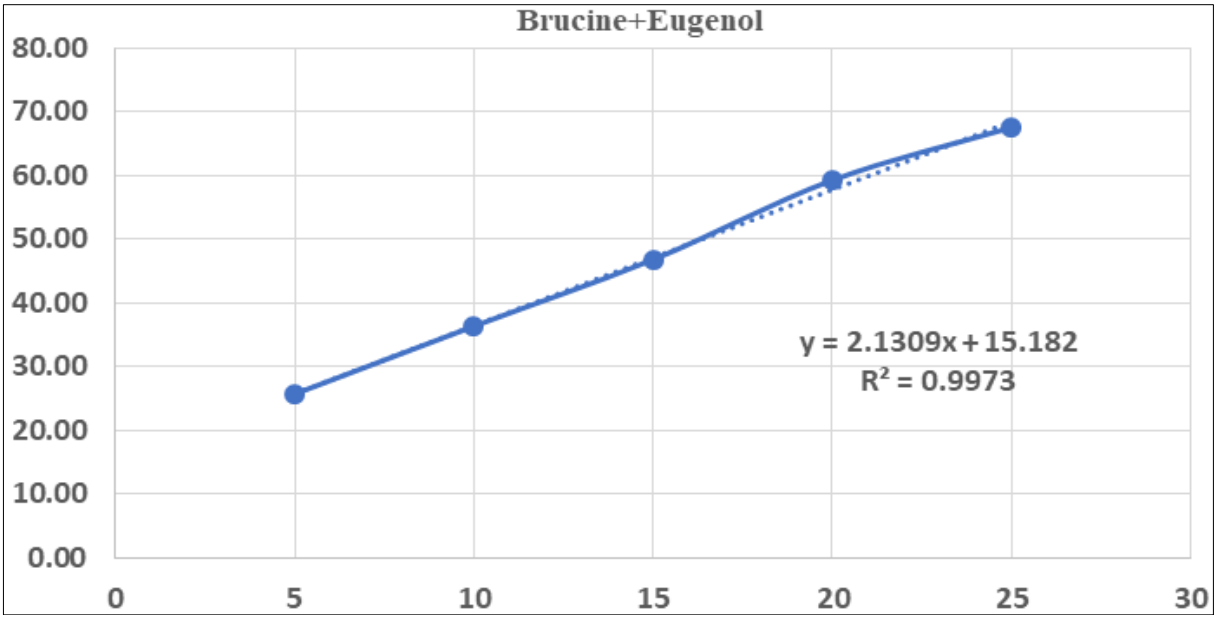
Graph 5: Ic_{50} value of Standard by FRAP method



Graph 6: Ic_{50} value of Brucine by FRAP method



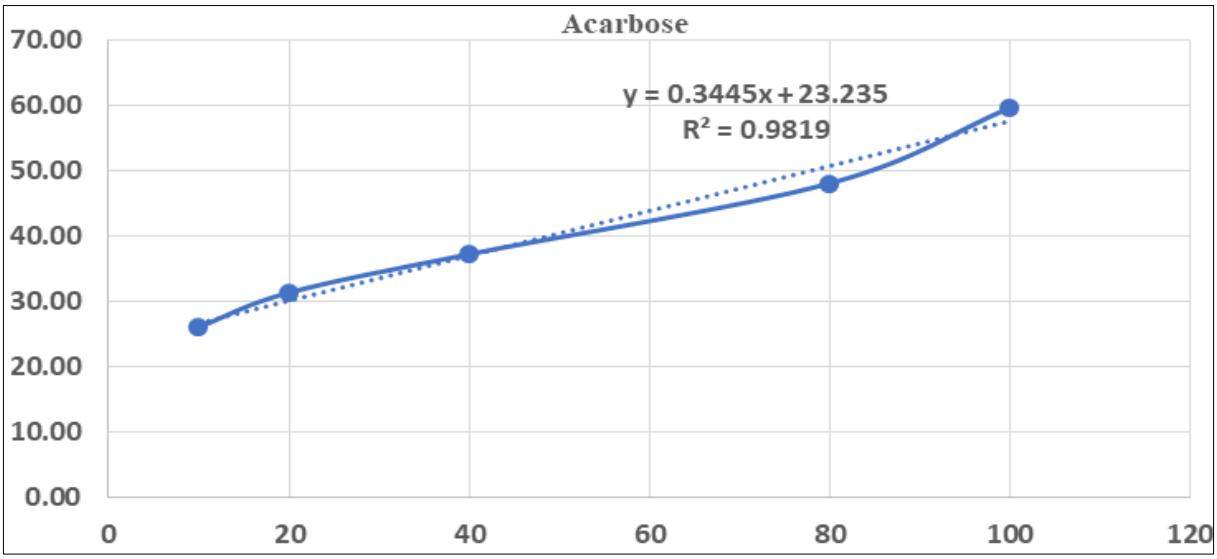
Graph 7: Ic_{50} value of Eugenol by FRAP method



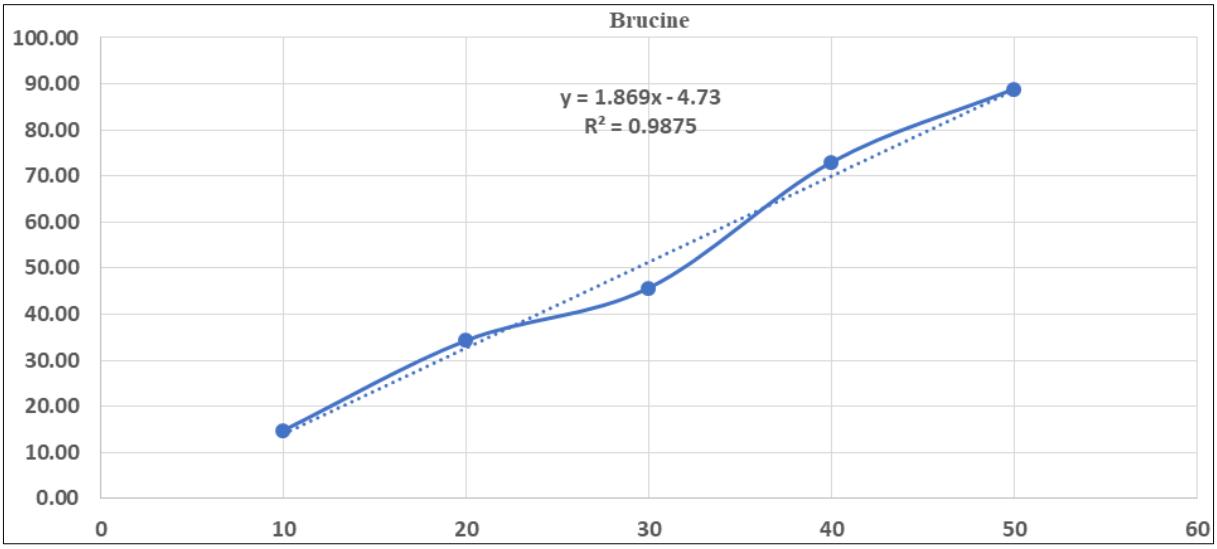
Graph 8: IC_{50} value of Brucine + Eugenol by FRAP method

Table 3: Inhibitory activity of standard, Brucine, Eugenol and Brucine + Eugenol against α-amylase

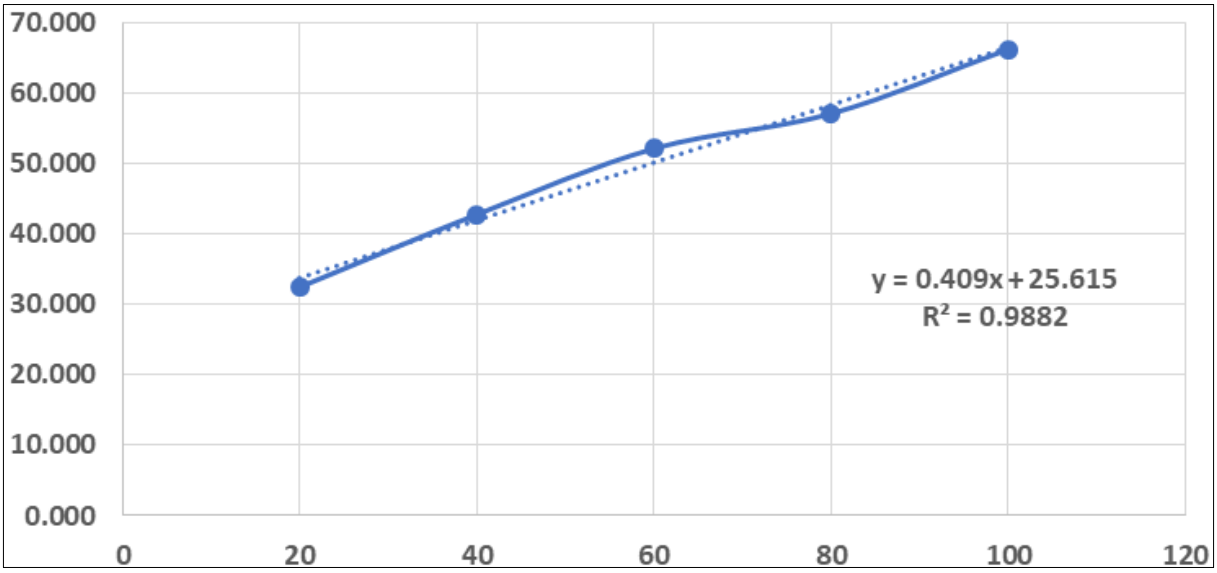
Drug	Concentration (µg /ml)	Abs of sample	Blank Abs	Sample - Blank	Control Abs	(Control Abs) (Sample - Blank)	(Control Abs)-(Sample-Blank)/ Control Abs	IC_{50}(µg/ml)
Acarbose	10	0.76	0.057	0.703	0.951	0.248	26.08	77.69±2.96
	20	0.71	0.057	0.653	0.951	0.298	31.34	
	40	0.654	0.057	0.597	0.951	0.354	37.22	
	80	0.551	0.057	0.494	0.951	0.457	48.05	
	100	0.441	0.057	0.384	0.951	0.567	59.62	
Brucine	10	0.86	0.057	0.803	0.951	0.148	14.80	29.28±1.15
	20	0.665	0.057	0.608	0.951	0.343	34.30	
	30	0.551	0.057	0.494	0.951	0.457	45.70	
	40	0.278	0.057	0.221	0.951	0.73	73.00	
	50	0.119	0.057	0.062	0.951	0.889	88.90	
Eugenol	20	0.699	0.057	0.642	0.951	0.309	32.492	63.48±3.96
	40	0.601	0.057	0.544	0.951	0.407	42.797	
	60	0.512	0.057	0.455	0.951	0.496	52.156	
	80	0.465	0.057	0.408	0.951	0.543	57.098	
	100	0.378	0.057	0.321	0.951	0.63	66.246	
Brucine + Eugenol	20	0.721	0.057	0.664	0.951	0.287	30.179	55.83±1.96
	40	0.612	0.057	0.555	0.951	0.396	41.640	
	60	0.498	0.057	0.441	0.951	0.51	53.628	
	80	0.409	0.057	0.352	0.951	0.599	62.986	
	100	0.317	0.057	0.26	0.951	0.691	72.660	



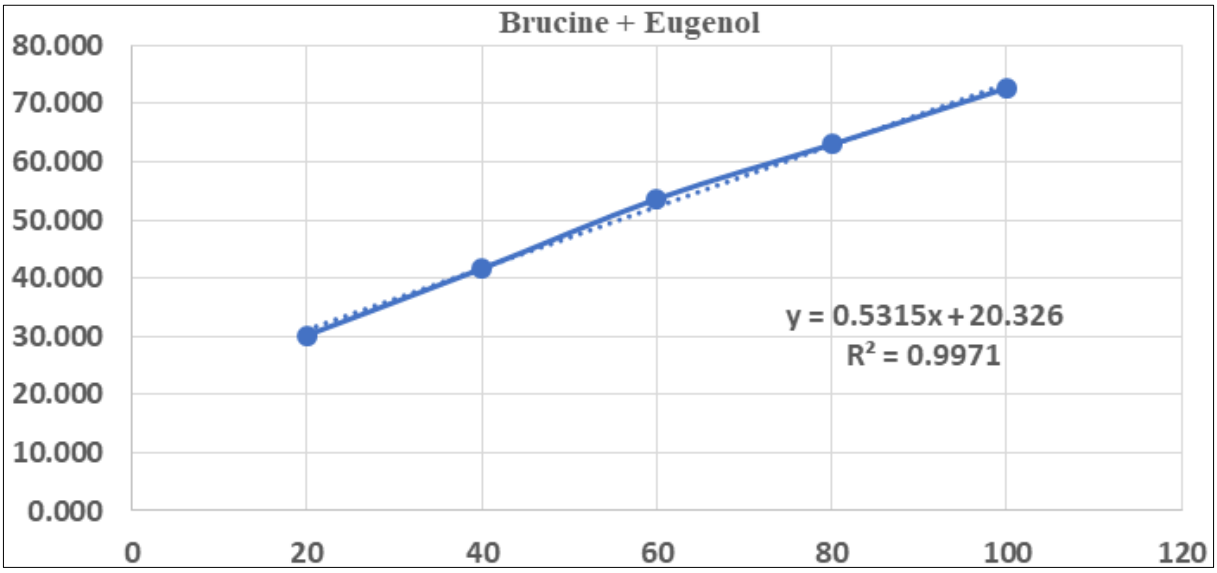
Graph 9: IC_{50} value of standard by α-Amylase method



Graph 10: Ic_{50} value of Brucine by α-Amylase method



Graph 11: Ic_{50} value of Eugenol by α-Amylase method



Graph 12: Ic_{50} value of Brucine + Eugenol by α-Amylase method

Discussion and Conclusion

The Results revealed that brucine and eugenol in combination possess significant anti-oxidant activity in different *In vitro* models and anti-diabetic activity through *In vitro* α-amylase method. The hydrogen peroxide method has IC $_{50}$ value as 39.52 µg/ml which is compared with

ascorbic acid as a standard (Table 1). In FRAP method has Ferrous reducing power has IC_{50} Values of 16.33 µg/ml which is compared with ascorbic acid as a standard in (Table 2). *In vitro* α-amylase inhibition activity has IC_{50} value of 55.83 µg/ml when compared to acarbose as a standard (Table 3). The IC_{50} values of Brucine and eugenol in combination has lesser than the individual effects. Hence it is synergistic to use in combination rather than individually. Various studies have shown that diabetes is associated with increased formation of free radicals, the phytochemical possessing both anti-diabetic and anti-oxidant activity can be great advantage in treatment of diabetes. *In vivo* studies, clinical trials and further study are necessary to elucidate the mechanism of action at cellular levels and components present in it.

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