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In-Vitro Antioxidant and Pharmacodynamic Potential of *Hibiscus tiliaceus* L. against Experimentally Induced Depression in Rats

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ABSTRACT

Objective: The rationale of the prevailing study was to assess the *in vitro* antioxidant and *in vivo* anti-depressant activity of ethanolic extract of *Hibiscus tiliaceus* L. **Methods:** The whole plant ethanolic extract of *Hibiscus tiliaceus* L. was obtained by the process of maceration. Preliminary phytochemical analysis and the acute toxicity studies were executed to analyze the susceptible parameters. This extract was evaluated for *in vitro* antioxidant properties by employing hydrogen peroxide scavenging assay and reducing power assay. The anti-depressant activity was carried out on rats using the most widely accepted behavioral models Force swim test (FST) and Tail swim test (TST). **Results:** Preliminary phytochemical analysis conceded the presence of substantial constituents like polyphenols, glycosides etc. The *in vitro* hydrogen peroxide scavenging assay and reducing power assay affirm the IC_{50} values of 15µg/mL and 28µg/mL respectively which are comparable to the standard. The extract IC_{50} values in FST and TST revealed the immobility period of 162 and 112 seconds respectively at 200mg/kg dose which are in comparison with the standard drug Sertraline with immobility time of 123 sec and 105 sec at a dose of 25mg/kg. The enhanced dose of extract, 400mg/kg acknowledged the inclined anti-depressant activity. **Conclusion:** The ethanolic extract of *Hibiscus tiliaceus* L. which was found to possess both anti-oxidant and anti-depressant activities can be a distinguished natural therapy for treating depression associated with oxidative stress.

INTRODUCTION

Globally by the year 2020, depression will turn out to be the second dominant cause of premature deaths and the most prevalent and existing complaint clinically. Oxidative stress may associate to psychiatric disorders and is being regarded as a feasible mechanism that can affect the regulation of illnesses [1]. The overproduction of ROS and oxidative stress have been implicated in the pathophysiological processes related to various diseases, including Alzheimer's, Parkinson's, anxiety, and depression [2]. Stressful conditions can shower anxiety and depression, which is inclined to the excessive production of free radicals, and in turn lead to oxidative stress. Adding on, the abnormal oxidative product levels were seen in blood, red blood cells (RBCs), urine and cerebrospinal fluid [3]. In depression, the CSF levels of metabolites of monoamines - serotonin, norepinephrine and dopamine are reduced. Choice of antidepressant agents mainly depends on cost and side effect profile [4]. Selective serotonin reuptake inhibitors (SSRIs) are used widely for treatment of depression. Although they are relatively safe and effective as antidepressants, SSRIs produce adverse reactions like somnolence, dizziness, elevation of liver enzymes, metabolic disturbances and sexual dysfunction [5]. Hence, the potential constituents of plants are used as alternatives for the treatment of oxidative stress-related diseases such as depression, owing to the presence of carotenoids, flavonoids, vitamins, and polyphenols [6, 7].

Currently, there are different types of treatments to which a large percentage of depressed patients respond, but the improvement is still disappointing. The reasons might be the various physiological side effects and tolerance on chronic treatment [8]. In the current scenario, we are in need of drugs with less side effects and more efficacy. In treating the neurological disorders, herbal medicines are extensively used due to their therapeutic efficiency and minimum side effects. Hence investigations have been progressed for the search of new and better tolerated molecules from plant sources, emphasizing the effectiveness of different plant species in a variety of animal models [9]. Extensive preclinical *in vitro* and *in vivo* studies have been conducted to validate phytotherapy of plants extracts [10].

Hibiscus tiliaceus Linn is a plant which belongs to the family Malvaceae, commonly known as "bola" exists as herb, shrub and around 250 species of same genus were found in tropical and subtropical regions of the world, out of which out 40 species occur in India [10]. Since ancient times Hibiscus species were used as folk remedies for various disorders. In folk medicine, the leaves of this plant used to treat fevers, soothe coughs, ulcer, wounds and

various skin diseases. Also, it was used for dry throat, ear infections, chest congestion, diarrhea, dysentery and typhoid [11]. The extract of *H. tiliaceus* was reported to have various interesting pharmacological activities such as antioxidant, anti-inflammatory, anthelmintic and antimicrobial activity [12, 13]. The various phytochemicals isolated from plant are hibiscus, hibiscus amide, vanillic acid, P-hydroxybenzoic acid, syringic acid, P-hydroxybenzaldehyde, scopoletin, N-trasferuloyltyramine, N-cis-feruloyltyramine, β-sitosterol, stigmasterol, β-stigmasterononе, hibiscolactone, hibiscones, hibiscoqinones, lapachol, gossypol, gossypetin, manosononеs, hyperoside, kaempferol, quercetin, gossypetin, gossytrine, para-coumaric and fumaric acid [14].

The objective of this study was to determine the *in vitro* antioxidant activity, the acute toxicity profile (LD_{50}) and to evaluate the effects produced by the acute administration of ethanolic extract of *Hibiscus tiliaceus* using the forced swim test and tail suspension tests to figure out the antidepressant activities of this medicinal plant.

MATERIALS AND METHODS

Collection of plant material

The whole plant of *Hibiscus tiliaceus* was collected from the local market in Kukatpally, Telangana, India which was identified and authenticated by Prof. Suresh Babu, Dept. of Botany, Govt. Degree College, Kukatpally, Hyderabad.

Preparation of extract

The whole plant material of *Hibiscus tiliaceus* was collected and then dried under shade for a period of three weeks. The dried plant material was milled to a fine powder using commercial laboratory blender and were stored in airtight containers until extraction. Maceration was a simple widely used procedure involves leaving the pulverized plant to soak in a suitable solvent in a closed container [15].

Simple maceration was performed at room temperature by mixing the ground drug with the ethanol solvent (drug solvent ratio 1:5 or 1:10). The mixture was retained for five days with occasional stirring. The extract is then repeated from the plant particles by straining. This process is repeated once or twice with fresh solvent. The residue of the extract was pressed out of the plant particles by vacuum filtration using a Buchner funnel. Vacuum filtration is

mainly used in order to dry the product in less time. The excess ethanol is recovered by distillation. The final residue obtained after filtration is ethanolic extract of *Hibiscus tiliaceus* (EEHT).

Tests for Preliminary Phytochemical Screening

Ethanolic extract of *Hibiscus tiliaceus*(EEHT) was subjected to preliminary phytochemical investigations to identify various phytoconstituents present in whole plant according to the following standard methods [16].

Test for alkaloids: A small portion of crude extract was dissolved in 5 ml of 1% hydrochloric acid, filtered and tested with Dragendroff's reagent and Mayer's reagent separately. Any precipitate or turbidity with the reagents suggested the presence of alkaloids.

Test for flavonoids: A few drops of conc. hydrochloric acid and 1-2 magnesium turnings were added to 1 ml of ethanolic extract. The presence of flavonoids was indicated by the development of pink or magenta-red color.

Test for phenols (Ferric chloride test): A fraction of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black color.

Test for amino acids and proteins (1 % Ninhydrin solution in acetone): 2 ml of filtrate was treated with 2-5 drops of ninhydrin solution placed in a boiling water bath for 1-2 minutes and observed for the formation of purple color.

Test for carbohydrates (Molisch test): To a fraction of EEHT, α-naphthol and alcohol were added. It was mixed well and conc. sulphuric acid was added drop by drop by keeping the test tube in inclined position. Violet ring is formed at the junction of two layers which indicates the presence of carbohydrates.

Test for saponins (Foam test): 2 ml of EEHT was added to 6 ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirmed the presence of saponins.

Test for sterols (Liebermann-Burchard test): 2 ml of EEHT was treated with drops of chloroform, acetic anhydride and conc. H_2SO_4 and observed for the formation of dark pink or red color.

Test for tannins (Braymer test): 2 ml of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish color solution.

Fixed oils and fats (Oily spot test): A drop of extract was placed on filter paper and solvent was allowed to evaporate. An oily stain on filter paper indicates the presence of fixed oil.

Test for proteins (Millon's test): The extract was treated with Millon's reagent. Appearance of pink color indicates the presence of proteins.

***IN VITRO* ANTI-OXIDANT ASSAY**

EEHT was tested for *in-vitro* antioxidant activity by using two standard methods, Hydrogen peroxide radical scavenging assay and reducing power assay. The absorbance was measured spectrophotometrically against corresponding blank solution. The percentage inhibition was calculated by the following formula.

$$\text{Radical scavenging activity (\%)} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \text{x} 100$$

IC_{50} which is the concentration of sample required to scavenge 50% of the free radicals was calculated. IC_{50} was calculated from equation of line obtained by plotting a graph of concentration versus percentage inhibition [17].

H_2O_2 radical scavenging assay

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate [17].

Preparation of reagents

Phosphate buffer solution pH 7.4: Add 250.0 ml of 0.2 M potassium dihydrogen phosphate to 393.4 ml of 0.1 M sodium hydroxide and make up the volume to 1000 ml with the distilled water.

Potassium dihydrogen phosphate (0.2M) solution: Potassium dihydrogen phosphate (2.72 g) was dissolved in distilled water and volume made up to 100 ml.

Sodium hydroxide solution (0.1M) solution: 0.4 g of sodium hydroxide was dissolved in distilled water and volume made up to 100 ml.

Procedure

A solution of hydrogen peroxide (2mmol/l) was prepared in phosphate buffer (P^H 7.4). The extract (10–50 µg/ml) was added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with ascorbic acid, the reference compound.

Reducing Power Assay

This method was based on the principle of increase in the absorbance of the reaction mixtures indicates an increase in the antioxidant activity. In this method, substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm [18].

Potassium ferricyanide + Ferric Chloride $\longrightarrow$Potassium ferrocyanide + Ferrous Chloride

Preparation of reagents

Phosphate buffer pH 6.6: Potassium dihydrogen phosphate (62.5 ml 0.2 M) was added to 250 ml volumetric flask and also 20.5 ml of 0.2 M NaOH and made upto volume 250 ml with distilled water.

Phosphate (0.2 M) solution: Potassium dihydrogen phosphate (2.72 g) was dissolved in distilled water and volume made up to 100 ml.

Sodium hydroxide solution (0.2 M) solution: 0.8 g of sodium hydroxide was dissolved in distilled water and volume made up to 100 ml.

Potassium ferricyanide (1% w/v) solution: Potassium ferricyanide (1g) was dissolved in water and volume made up to 100 ml in volumetric flask.

Ferric chloride solution (0.1% w/v): Ferric chloride (25 mg) was dissolved in distilled water and volume made up to 25 ml in volumetric flask.

Procedure

To 1 ml of EEHT and standard compound, add 2.5 ml of potassium ferricyanide (1 % w/v), 2.5 ml of phosphate buffer (pH 6.6) and incubate at 50^0C for 30 min.To 2.5 ml of above supernatant liquid, added 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ solution (0.1% w/v).The absorbance of ferric ferrous complex was measured using phosphate buffer pH 6.6 as control at 700 nm using UV-Visible spectrophotometer and estimated the enhanced absorbance values.

ACUTE TOXICITY STUDIES OF EEHT

Acute toxicity studies were performed according to the OECD 425 guidelines. Female Wister rats weighing 100-150g were selected and divided into four groups containing three animals in a group. Depending on the mortality or morbidity of animals a few steps may be necessary to judge the toxicity of the test substance. Minimal usage of animals while it allows for acceptable data is the main advantage of this method. The single dose of the ethanolic extract starting from 5mg/kg up to 2000mg/kg (5, 50, 300, 2000 mg/kg) was administered orally. The starting dose of the ethanolic extract of *Hibiscus tiliaceus* L was 200 mg/kg (p.o). The drug treated animals were carefully observed individually for the toxicity signs and mortality up to14days [19].

ANIMALS

Animal Protocol was approved by IAEC (Institutional Animal Ethical Committee) of CPCSEA (Committee for Purpose of Control and Supervision of Experimentation on Animals) through its reference no: IAEC/SVCP/2016/005, Dated: 27/2/16. Wistar rats, weighing (180-250 gms) were obtained from NIN (National Institute of Nutrition, Hyderabad). The animals were acclimatized to the experimental room at a temperature of 23 ± 2^0 C, controlled humidity conditions (50-55%) and 12 hr light and 12 hr dark cycles. They were fed with standard food pellets (Hindustan Lever, Hyderabad) and water *ad libitum*.

Experimental protocol:

Wister rats of either sex weighing between 160-250 gms were divided into four groups of six rats each (n=6).

Group I	Control (Saline, 0.9% NaCl)
Group II	Standard (Sertraline 25 mg/kg, p.o.)
Group III	Ethanolic extract of *Hibiscus tiliaceus* (200 mg/kg, p.o.)
Group IV	Ethanolic extract of *Hibiscus tiliaceus* (400 mg/kg, p.o.)

In the behavioral screening, animals were observed at fourteen days consecutive after oral route (p.o.) administration of EEHT (200 and 400 mg/kg, p.o.). During fourteen consecutive days, the animals were observed to detect general signs of toxicity piloerection, prostration, writhing, increased evacuation, grooming, discrete groups, dyspnea, sedation, analgesia and palpebral ptosis. All the animals were subjected to the forced swim test (FST) and tail suspension test (TST) after 30 min of the last dose of treatment.

ANTI-DEPRESSANT ACTIVITY

Forced swim test (FST):

The FST is a pharmacological method used for determination of antidepressant potential in animal models. The rats were forced to swim in a restricted space from which they cannot escape, and animals were selected based on their characteristic behavior of immobility. This behavior reflected a state of despair which can be reduced by using several agents which are therapeutically effective in human depression. Rats of either sex were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25±1ºc. The first group was assigned as control received only saline. Second group received standard drug (sertraline 25 mg\kg), third and fourth groups received EEHT (200 mg\kg and 400 mg\kg). In total time period of 8 min, the duration of immobility was recorded during the last 5 min. After an initial 3 min period of vigorous activity, each animal assumed a typical immobile posture. A rat was considered to be immobile when it remained floating motionless in the water, ceased for struggling and making only minimum movements of its limbs necessary to keep its head above water. Changes in duration of immobility of each group were studied in this model. A decrease in the duration of immobility is indicative of an antidepressant like effect [20].

Tail suspension test (TST)

The tail suspension test was performed according to the established procedure on rats. Mice were suspended on the edge of the table, 50 cm above the floor, with the help of an adhesive tape placed approximately 1 cm from the tip of the tail. The total duration of immobility induced by tail suspension was recorded for 6 min from the total of 10 min period. Animal was considered to be immobile when it did not show any movement of the body, hanged passively and completely motionless. The duration of immobility was noted by a trained observer for 5 min [21].

Statistical Analysis

The results are expressed as Mean±SEM. The statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison method. p values less than 0.05 were considered as significant.

RESULTS

Percentage yield of the extract

The yield acquired from the whole plant ethanolic extract of Hibiscus tiliaceus (EEHT) was about 50g from 1000g of dried leaves. Hence the percentage yield was found to be 5%.Phytochemical Screening.

The EEHT was screened preliminarily for the presence of various categories of phytoconstituents reported in Table No. 1.

Table No. 1: Phytochemical constituents of ethanolic extract of *H. tiliaceus*

Sr. No.	Test for Phytochemical Constituents	Reagent	Result
1	Alkaloids	Mayer's Test	-
		Dragendroff's Test	-
		Wagner's Test	-
		Hager's Test	-
2	Glycosides	Borntrager's Test	+
		Keller-killiani Test	+
3	Triterpenoids	Liebermann's Test	+
		Liebermann-Buchard Test	+
4	Flavanoids	Shinoda Test	+
5	Saponins	Frothing Test	+
6	Reducing Sugars	Molish Test	-
7	Tannins	Ferric Chloride Test	+
		Potassium dichromate Test	+
		Lead acetate test	+
8	Proteins	Biuret Test	-
9	Fixed oils and fats	Oily Spot Test	-

+ = Present, - = Absent

The preliminary analysis of phytoconstituents indicated that the EETH is enriched with glycosides, triterpenoids, flavonoids, saponins and tannins, contributing for various pharmacological activities of the plant.

IN-VITRO ANTIOXIDANT ACTIVITY

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging efficiency of EEHT, depicted in Table No. 2 revealed the dose dependent inhibition of peroxide radical. The activity of the ethanolic extract of the plant enhanced prominently with the upsurge in the concentration. EEHT elicited the competent inhibition of hydrogen peroxide with 95.6% at a maximum concentration of 50 µg/ml which is proportionate to the standard percent inhibition of 98.05% at identical

concentration. The IC_{50} values of EEHT and the standard emerge to be proximate to each other with 54 µg/ml and 66.1 µg/ml respectively (Table No. 2).

Table No. 2: Effect of EEHT on Hydrogen peroxide scavenging assay

Sr. No.	Test extract	Dose (µg/mL)	% inhibition (Mean±SEM)	IC_{50}value (µg/mL)
1	Ascorbic acid (Standard)	10	45.25 ± 0.24	54±0.01
		20	52.4 ± 0.39	
		30	78.8 ± 0.79	
		40	90.7 ± 0.69	
		50	95.6 ± 0.59	
2	EEHT	10	42.9 ± 0.89	66.1±0.06*
		20	57.5 ± 0.79	
		30	77.35 ± 0.34	
		40	85.9 ± 0.59	
		50	98.05 ± 0.59	

*$p<0.01$ considered as significant; compared with corresponding standard

Reducing power assay

EEHT was assessed for the reduction potential ability to react with potassium ferricyanide (Fe^{+3}) and reduce it to Fe^{+2} form. It was found that absorbance with increased concentration significantly increased the reducing power. EEHT reduced the generation of free radicals, showed the absorbance of 1.42 indicated good ($p<0.01$) reducing ability power which was comparable to Ascorbic acid.

Table No. 3: Absorbance of test extract and standard Ascorbic acid at various concentrations in Reducing Power Assay

Sr. No.	Test extract	Dose (µg/ml)	Absorbance (Mean ± SEM)
1	Ascorbic acid (Standard)	10	0.36 ± 0.01
		20	0.51 ± 0.03
		30	1.06 ± 0.04
		40	1.62 ± 0.02
		50	1.89 ± 0.03
2	EEHT	10	0.24 ± 0.01
		20	0.36 ± 0.03*
		30	0.65 ± 0.02*
		40	1.12 ± 0.01*
		50	1.42 ± 0.01*

*p<0.01 considered as significant; compared with corresponding standard

ACUTE TOXICITY STUDIES

Acute toxicity profile of EEHT, performed as per the OECD guideline, revealed that the lethal dose (LD_{50}) of the extract is greater than 2000mg/kg body weight. Different doses of EEHT were administered to 12 rats grouped into four. Group I received 5mg/kg, Group II-50mg/kg, Group III-500mg/kg and Group IV-2000mg/kg dose of the extract. Parameters observed for the span of 14 days include motor activity, tremors, convulsions, straub reaction, piloerection, sedation, muscle relaxation, hypnosis, analgesia, ptosis, lacrimation, diarrhea, restlessness, circling movements and change in the skin color (Table No. 4). This study indicated that there are no signs of toxicity or mortality noticed up to a dose of 2000mg/kg and hence the LD_{50} was considered to be greater than 2000mg/kg.

Table No. 4: Effect of EEHT on acute toxicity test in rats

S.NO	Parameter observed	Animals			
		After treatment 3mg/kg	After treatment 50mg/kg	After treatment 500mg/kg	After treatment 2000mg/kg
1	Motor activity	NE	NE	NE	NE
2	Tremors	NE	NE	NE	NE
3	Convulsion	NE	NE	NE	NE
4	Straub reaction	NE	NE	NE	NE
5	Piloerection	NE	NE	NE	NE
6	Sedation	NE	NE	NE	NE
7	Muscle relaxation	NE	NE	NE	NE
8	Hypnosis	NE	NE	NE	NE
9	Analgesia	NE	NE	NE	NE
10	Ptosis	NE	NE	NE	NE
11	Lacrimation	NE	NE	NE	NE
12	Diarrhea	NE	NE	NE	NE
13	Restlessness	NE	NE	NE	NE
14	Circling movements	NE	NE	NE	NE
15	Change in skin color	NE	NE	NE	NE

NE- No Effect

ANTI-DEPRESSANT ACTIVITY

Forced swim test (FST)

In FST, the extent of immobility is correlated with that of control and the anti-depressant drug Sertraline which is employed as standard. The EEHT when given in the expanded doses of 200 and 400mg/kg originated the momentous rebate in the duration of immobility with 162sec and 143 sec respectively (Table No. 5). These values exhibited by EEHT were quite

less than the control (181sec) and are proximate to that of the standard I.e. 123sec. These values suggest the efficient anti-depressive activity of EEHT.

Table No. 5: Effect of EEHT on duration of immobility in FST and TST

Groups	Drug treatment	Dose	FST Immobility period (Sec)	TST Immobility period (Sec)
1	Control	(0.9% NaCl)	181 ±3.5	168 ±1.5
2	Sertraline (Std)	25mg/kg	123±4.6	105±4.7
3	EEHT	200mg/kg	162 ± 5.7**	112 ± 2.7**
4	EEHT	400mg/kg	143 ± 8.3**	124 ± 6.9**

Values expressed in Mean±SEM (n=6), **p<0.001 compared with control and standard treated animals.

Tail Suspension Test (TST)

EEHT exhibited significant declinement in the immobility period in TST at a dose of 200mg/kg and 400mg/kg with the span of 112sec and 124sec respectively. These values are distinguished with that control and the standard drug sertraline and are considered to be prominent. Results were tabulated in Table No 5.

DISCUSSION

Depression, a mental disorder characterized by psychological distress is posing its alarming spread by crawling from fourth to second most frequently caused disease by 2030 [22]. This is defining the immediate need to investigate and develop novel antidepressant drugs. Though many classes of efficient anti-depressant drugs are helping in maintaining the symptoms of the disease, they have handful of side effects which proposes another challenge for treatment [23, 24].

Another noteworthy ailment that grabbed the attention, contributing as one of the major causes of depression is oxidative stress i.e., deficiency of anti-oxidant properties [25]. A meta-analysis has reported that anti-oxidants supplementation has got potential benefits in treating depression [26]. A recent study report in 2019 also revealed that decreased intake of polyphenol compounds and other nutrients results in depression [27].

Hence, the natural products with fewer side effects began to get explored for treating depression. The EEHT was found to possess ant-oxidant properties and also exhibited efficient inhibition of immobility period in the most widely accepted models for assessing depression, the FST and TST. The extract also elicited the presence of polyphenol compounds like flavonoids and therefore can be a good natural therapy for treating depression.

CONCLUSION

EEHT, proved to be an exemplary extract in treating depression by neutralizing reactive oxygen species and eliciting magnificent effect in the anti-depressant models, needs further exploration of individual constituents responsible for their role in dealing with oxidative stress and depression.

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