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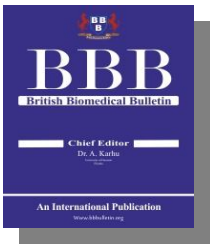

Original

In vitro Antioxidant and *In vivo* Nootropic Activity of Ethanolic Leaf Extract of *Ficus racemosa* Against Stress Induced Amnesia in Albino Rats

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ABSTRACT

The aim of the present study was to evaluate the Invitro Antioxidant and neuropharmacological parameters of the Ethanolic leaf extract of *Ficus racemosa* against stress induced amnesia in Albino rats. Nootropics are agents that enhance the cognitive skills. Learning and memory can be conceived as both a psychological process, as well as a change in synaptic neural connectivity. In vitro antioxidant activity was evaluated using the parameters such as Nitric oxide-scavenging activity, Hydrogen peroxide scavenging and Hydroxyl radical scavenging. Ascorbic acid with same concentration was used as a standard antioxidant. In the present study the extract (150 and 300 mg/kg, p.o) was investigated for Nootropic activity in normal and stress induced rats. Conditioned avoidance response using Cook's pole climbing apparatus and transfer latency using elevated plus maze were used in normal and stress induced rats to assess cognitive-improving activities. Daily administration of *Ficus racemosa* at doses of 150 and 300 mg/kg,p.o enhances cognition in dose dependent manner in normal rats. Fast retrieval was observed in extract treated stress induced rats, compared to that of stress control group.The study confirmed the Nootropic (cognition enhancement) activity of extract and the reported activity may be attributed to its antioxidant property.

Introduction

Generation of free radicals causes cumulative damage of DNA, proteins, lipids led to oxidative stress. This oxidative stress has been suggested to be the cause of ageing

and various human diseases like cancer, hepatic disorders, and diabetes [1]. DNA damage mediated by free radicals may result in mutation or chromosomal aberrations leading to carcinogenesis [2] .The use of medicinal herb in the treatment and prevention of diseases is attracting attention by scientists worldwide. Active oxygen species and free radicals play an important role in the initiation and evolution of numerous diseases. The use of compounds with antioxidant activity is expected to be useful for the treatment of these diseases. Therefore, there has been a growing interest in finding novel antioxidants in order to meet the requirements of pharmaceutical industries [3]

The human brain is almost certainly the least understood of our organs. When facing the diseases affecting the brain, the medical sciences are in an unfortunate situation of studying and attempting to prevent/cure unknown pathological processes where even the normal conditions are poorly understood. Alzheimer's disease, primarily affects the elderly population, and is estimated to account for 50-60% of dementia cases in persons over 65 years of age.[4]

Memory is ability of an individual to record event, information and retains them over short or long periods of time and recalls the same whenever needed. Age, stress and emotion are conditions that may lead to memory loss, amnesia, anxiety, high blood pressure, dementia, more ominous threat like schizophrenia and Alzheimer's diseases."Nootropics" are agents that enhance the cognitive skills. Learning and memory can be conceived as both a psychological process, as well as a change in synaptic neural connectivity. Cognitive deficits have long been recognized as severe and consistent neurological disorders associated with numerous psychiatric and neurodegenerative states. [5]

Before the development of modern medicine people relied on natural remedies for the treatment of CNS related maladies. It is on this basis that researchers keep on working on medicinal plants in order to produce/develop medicines from herbs, nutraceuticals or life style changes for controlling age related neurodegenerative disorders.

The plant used in the present study is *Ficus racemosa* which was evaluated for Invitro Antioxidant and invivo Nootropic Activity against stress induced amnesia in albino rats.

Materials and Methods

Plant collection

The leaves of *Ficus racemosa* were collected from the surroundings areas of Pithapuram and the plant was authenticated by Dr.T.V.Raghava rao, a leading taxonomist in Maharani College Peddapuram.

Preparation of Ethanolic extract of *Ficus racemosa* leaves

The freshly collected leaves of the plant *Ficus racemosa* were cleared from dirt and then the leaves were dried under shade for about 15 days and then coarsely powdered in a mechanical grinder .the powder was macerated with ethanol for 5 days ,filtrate was collected and concentrated. The leaves powder was packed in a soxhlet apparatus and extracted with 95% alcohol for 18 hours. Appearance of colorless solvent in the siphon tube was taken as the termination of the extraction .The extract was then transferred into a previously weighed beaker and evaporated to a thick paste on the water bath, maintained at 50^0C to get alcoholic extract. The concentrated product was dried using desiccators with anhydrous calcium chloride.

Chemicals and equipment

All the chemicals used are of analytical grade. Ascorbic acid was a gifted sample from GlaxoSmithKline. Ferric chloride, Sodium nitroprusside, sulphanilamide, Phosphoric acid, Napthylethylenediamine dihydrochloride, Hydrogen peroxide, sodium salicylate has been procured from Sigma chemicals.

The different glassware used for the experimental purpose is of standard quality. UV-Visible double beam (ELICO SL 210) and shimadzu electronic balance were used for the analysis.

In vitro antioxidant activity

Nitric oxide generation and assay of nitric oxide scavenging

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction as described previously. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide [6]. Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with different concentrations of the extracts (0.05, 0.1, 0.3 and 0.5 mg/ml) dissolved in the suitable solvent systems and incubated at 25 °C for 150 min. The sample above was reacted with Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% Napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme was read at 546 nm and referred to the absorbance of standard solutions of ascorbic acid was treated in the same way with Griess reagent. All the tests were performed in triplicate and the results averaged. Ascorbic acid used as the reference compound. The percentage decrease in absorbance was calculated.

Hydroxyl radical scavenging assay

The scavenging ability of the five sample extracts on hydroxyl radicals was determined according to the method described with some modifications[7]. Briefly, individual sample extracts (1 mL) at different concentrations (0.05,0.1,0.3 and 0.5 mg/ml) was added to the reagent containing 1 mL 1.5 mM $FeSO_4$, 0.7 mL 6 mM H_2O_2 and 0.3 mL 20 mM sodium salicylate. After incubation for 1 h at 37°C, absorbance of the reaction mixture was read at 562 nm. The scavenging ability on hydroxyl radicals was calculated.

Scavenging of hydrogen peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to our recently published papers [8]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (0.05, 0.1,0.3 and 0.5 mg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds were calculated .

Nootropic activity

Experimental animals

Albino rats weighing between 80-120g of either sex were used for the study. The animals were acclimatized to the laboratory environment for at least a week before experimentation. Food but not water

was deprived overnight and during the experiment. All the experiments were carried out during the light period (9:00-16:00 h). Each group consists of 4 animals and after learning/training each particular group was divided into two sub groups of 2 animals each. (One sub group without inducing stress & other sub group with inducing stress). The institutional animal ethical committee approved the study protocol .

EXPERIMENTAL DESIGN

Groups

Before training/learning

Group I: Animals (n=4) were administered with vehicle

Group II: Animals (n=4) were administered with lower dose (150mg/kg body weight) of Ethanolic leaf extract of *Ficus racemosa*

Group III: Animals (n=4) were administered with higher dose (300mg/kg body weight) of Ethanolic leaf extract of *Ficus racemosa*

After training

After training each group divided into two sub groups. One sub group subjected to stress other sub group left normally without any stress. The administration of vehicle/drug was normal as usually during this period.

Stress procedure

The animals were subjected to chronic mild stress using following protocol. The rats were forced to swim in a cylindrical vessel of height 60 cm and diameter 45 cm containing water at room temperature (28^oC). Water depth was maintained at 40 cm. the swim stress was conducted for about 15 min and 4 times a day about a week; the animals are placed on small platform (3 cm height&3.5cm diameter) fixed at center of chamber and surrounded by water 2 cm depth at 22^0c for 24 hrs, deprived of food and water during night, fallowing 3hr access to restricted food and 2 hr access to an empty water bottle in alternate days about a week.

Experimental procedure

Conditioned avoidance response (CAR) using Cook's Pole Climbing Apparatus

The Nootropic activity of Ethanolic root extract of *Ficus racemosa* in normal and stress induced rats was evaluated by using the conditioned avoidance response (*CAR*) [9]. Rats were initially divided into 3 groups each containing 4 animals. Groups II and III were administered orally with 150 and 300 mg/kg body weight respectively of *Ficus racemosa extract*, while animals in group I which received distilled water orally served as control. After 60 minutes of drug administration, all the animals were subjected to a training schedule individually by placing inside the Perspex chamber of the apparatus.

After an accustomed period of five minutes to the chamber, a buzzer was given followed by a shock through the grid floor. The rat had to jump on the pole to avoid foot shock. Jumping on the pole functionally terminates the shock and this was classified as an escape while such jumping prior to the onset of the shock was considered as avoidance. The session was terminated after completion of 30 trials with an interval of 20–30 seconds given for each trial. This procedure was repeated at 24 h intervals until each subject of each group reached 95 to 99% avoidance.

After attaining complete training of a particular group, the animals were divided into two sub groups containing 2 animals each. Then stress was induced to one of the sub group of a particular group for about week and another subgroup in each particular group were left normally.

Drug/vehicle administration is as usual during the period of stress. Again on 7th day retention of conditioned avoidance response was checked in both normal and stress induced groups. The training schedule was continued further with the daily doses of the extract/vehicle until they returned to normal level from stress induced amnesia.

Transfer Latency Using Elevated plus Maze Test

Rats were initially divided into 3 groups each containing 4 animals. Groups II and III were administered orally with 150 and 300 mg/kg body weight respectively of *Ficus racemosa extract*, while animals in group I which received distilled water were served as control. The maze was elevated to the height of 60 cm. rats were placed individually at the end of an open arm facing away from the central platform and the time it took to move from the end of open arm to either of the closed arms (transfer latency, TL) was recorded.

On the 1st day, rats of each group placed one by one individually on one of the open arm of the elevated plus maze and transfer latency was recorded. After measurement of Transfer latency they were allowed to explore the maze for about 5 min. On the 2nd day rats were placed again on the elevated plus maze as before and Transfer latency was noted again. [10]

After learning on 2nd day each particular group was divided into two sub groups as normal and stress groups. Then each subgroup of particular group subjected to stress from 2nd day to 9th day for about 7 days. On 9th day again transfer latency was checked for all the sub groups:

Finally inflexion ratio was calculated using the fallowing formula

$$Inflexion\ ratio = \frac{T.L\ \ on\ 1st\ day - T.L\ on\ 2nd\ \ (or)\ 9th\ day}{T.L\ on\ 2nd\ (or)\ 9th\ day}$$

Here T.L indicates transfer latency

Transfer latency and inflexion ratio on 2nd day indicates the learning capacity of rats while transfer latency and inflexion ratio on 9th day indicates their retention of memory.

Results & Discussion

OH radical is the most reactive free radical in biological systems and it can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions, such as copper and iron. Hydroxyl radical has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. For example, OH-radicals react with lipid, polypeptides, proteins and DNA, especially thiamine and guanosine. This radical has the capacity to conjugate with nucleotides in DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity [11]. The highly reactive OH radicals can cause oxidative damage to DNA, lipids and proteins [12]. As is the case for many other free radicals, OH radicals can be neutralised if it is provided with a hydrogen atom. The results in **Table 1** indicate that Ethanolic leaf extract of *Ficus racemosa* had strong hydroxyl radical scavenging activity similar to that of Ascorbic acid.

H_2O_2 is highly important because of its ability of penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells [13] Thus, removing H_2O_2 is very important for the protection of living systems. The results **Table 1** indicate that Ethanolic leaf extract of *Ficus racemosa* had strong hydrogen peroxide radical scavenging activity similar to that of Ascorbic acid.

Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by Ethanolic leaf extract of *Ficus racemosa* and Ascorbic acid.

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide[14,15]**.** Which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent, Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Ethanolic leaf extract of *Ficus racemosa* had comparably more nitric oxide radical scavenging activity than Ascorbic acid **Table 1**.

Conditioned Avoidance Response (CAR) Using Cook's Pole Climbing Apparatus

The Conditioned avoidance response of rats administered with the Ethanolic leaf extract of *Ficus racemosa* or vehicle increased gradually to 95-99% over seven to eleven days. The percent avoidance was always higher in the extract treated groups compared to vehicle treated control group. The acquisition (time to achieve 95% *CAR*) for the extract treated groups (150 & 300mg/kg) was quicker (9 & 7 days respectively) when compared to control (11 days) and the results were found to be dose dependent.

Reduction in observed CAR after induction of stress is a clear indication of stress induced amnesia. However, continued treatment of *Ficus racemosa* produced better retention and recovery in a dose dependent manner than the vehicle treated animals in stress induced groups. There was a less fall in mean percentage of CAR and recovery in extract treated stress induced subgroups compared to vehicle treated stress control group. But in normal sub groups (which were not subjected to stress) there is no significant reduction in CAR. Results were given fig 1,2. Group I (control) was showed maximum possible avoidance out of 30 trails in 11 day. In the normal subgroup of this group which left normally without stress about a week, the maximum recovery was seen on 20th day. In the stress subgroup of this group which were subjected to stress about a week, the recovery was seen on 22nd day. Group II (150mg/kg) was showed maximum possible avoidance out of 30 trails in 9 day. In the normal subgroup of this group which left normally without stress about a week, the maximum recovery was seen on 18th day. In the stress subgroup of this group which were subjected to stress about a week, the recovery was seen on 20th day. Group III (300mg/kg) was showed maximum possible avoidance out of 30 trails in 7th day. In the normal subgroup of this group which left normally without stress about a week, the maximum recovery was seen on 16th day. In the stress subgroup of this group which were subjected to stress about a week, the recovery was seen on 18th day.

The Stress plays a major role in various pathophysiological processes associated with neurodegenerative diseases &mental disorders[16]. There is increasing evidence that free radical–induced oxidative stress damage may play a role in the pathogenesis of Alzheimer's disease.[17,18] The cook's pole climbing apparatus was originally designed to find tranquillizing activity of substances. It has provision to find unconditioned response of the animal (climbing of pole after applying foot shock i.e., procedural learning) and also conditioned avoidance response of the animal (climbing of the pole after buzzer with significant training i.e., declarative training). Elevated plus maze was used to measure the anxiety state in animals, however transfer latency was markedly shortened if the animal had previous experience in entering open and closed arms, and this shortened transfer latency has been shown to be related with memory processes. Recent studies of several nootropics and amnestic agents on elevated plus maze made this model a widely accepted paradigm to study learning and memory processes in rodents. It was useful for the evaluation of effect of drug on both acquisition and retention of memory phases.

The antioxidant and anti stress activity were correlated with the nootropic activity of the extract since the role of stress and free radicals have been implicated in the loss of memory, concentration & also in Alzheimer's disease [19,20]**.** Earlier reports indicate that the antistress activity of some potential medicinal plants could be attributed due to their antioxidant effects[20]. So the antioxidant activity of *Ficus racemosa extract* was studied. From the results **Table 2,3** it was found that *Ficus racemosa* extract has significant antioxidant activity comparable to standard Ascorbic acid.

Conclusion

The present study demonstrates scientific support for the protective effect of ethanolic root extract of *Ficus racemosa* to combat stress induced amnesia and lends some credence to traditional claims of its therapeutic benefits in stress and stress-related disorders.

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Table 1. *In vitro* Antioxidant potential of Ethanolic Leaf extract of *Ficus racemosa* and ascorbic acid against Nitric Oxide, Hydroxyl and H_2O_2 radicals

Conc. (mg/ml)	Nitric oxide (%)		Hydroxyl (%)		H_2O_2 (%)	
	ETH	STD	ETH	STD	ETH	STD
0.05	52.71±0.35	58.45±0.2 3	33.02 ±0.18	69.47±0.32	32.84±0.25	51.22±0.32
0.1	63.73±0.18	70.07±0.22	42.32±0.25	82.55±0.26	48.48±0.29	60.08±0.36
0.3	70.45±0.13	71.76±0.32	57.44±0.36	83.46±0.12	56.04±0.15	65.15±0.19
0.5	75.29±0.13	74.84±0.13	70.02±0.21	87.65±0.16	69.20±0.25	75.19±0.18

Table 2. Effect of Ethanolic leaf extract of *Ficus racemosa* on Transfer latency(in sec) on 2^{nd} and 9^{th} day in normal rats using elevated plus maze

Treatment	Transfer latency (in sec)			Inflexion ratio	
	Day 1	Day 2	Day 9	Day 2	Day 9
Control	60.50±3.52	35.5±2.67	20.50±2.09	0.70	1.95
F.racemosa (150mg/kg)	36.50±2.32	20.0±3.21	10.50±3.23	0.82	2.47
F.racemosa (300mg/kg)	42.50±3.21	22.0±2.61	11.50±2.12	0.93	2.69

All the values are expressed as mean ±SEM

Table 3. Effect of Ethanolic leaf extract of *Ficus racemosa* on Transfer latency(in sec) on 2nd and 9th day in stress rats using elevated plus maze

Treatment	Transfer latency (in sec)			Inflexion ratio	
	Day 1	Day 2	Day 9	Day 2	Day 9
Control	60.50±3.52	35.5±2.67	25.50±1.02	0.70	1.37
F.racemosa (150mg/kg)	36.50±2.32	20.0±3.21	18.50±1.23	0.82	0.97
F.racemosa (300mg/kg)	42.50±3.21	22.0±2.61	15.50±2.10	0.93	1.74

All the values are expressed as mean ±SEM

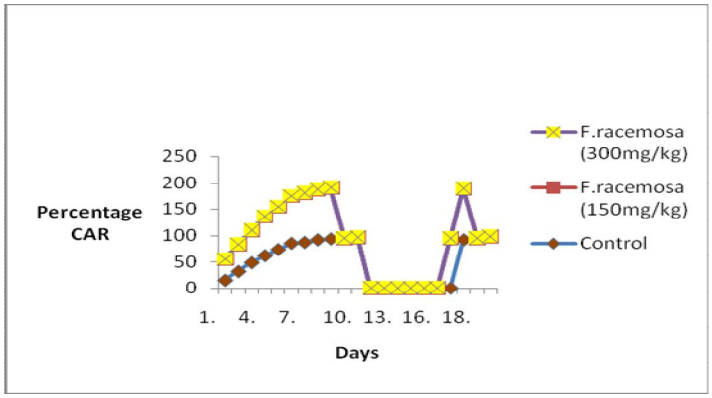

Figure 1. Comparison of mean percentage of conditioned avoidance response (CAR) out of 30 trails in normal sub group rats of different groups

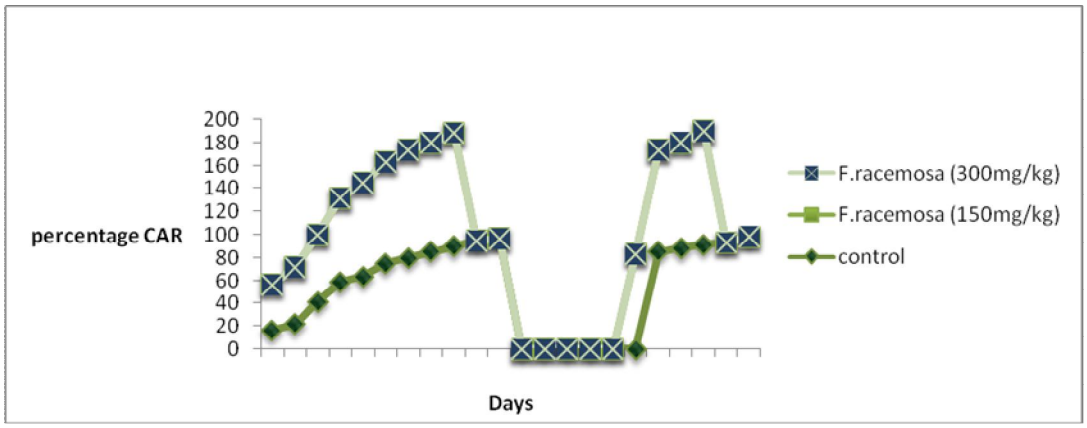

Figure 2. Comparison of mean percentage of conditioned avoidance response (CAR) out of 30 trails in stress sub group rats of different groups