Keywords: Antistress activity; Ficus benghalensis; Forced swimming test; Tail suspension test; Elevated plus maze; Anoxic tolerance test; Pentylenetetrazol-induced convulsion; Writhing test

ABSTRACT

Aim: The present study was undertaken to investigate the antistress potential of isolated flavonoids rutin and quercetin from Ficus benghalensis. Materials and Methods: Male Swiss albino mice were treated with different doses of the isolated flavonoids rutin and quercetin (i.e. 80 and 100 mg/kg orally) and behavior was observed on the Forced swimming test, Tail Suspension Test, Elevated plus maze model, Anoxic tolerance test, Pentylenetetrazol-induced convulsion and Writhing test. Results: Mice pretreated with rutin and quercetin both at the doses of 80 and 100 mg/kg showed significant improvement in the swimming time and showed a decrease in immobility time in swimming endurance. Increase in anoxic tolerance time was observed in anoxic tolerance test when animals were treated with rutin and quercetin. On the other hand, after administration, rutin and quercetin significantly increased the time spent in open arm and decreased the time spent in the closed arm compared to the control group in elevated plus maze model. The effect of glacial acetic acid was also reduced by flavonoids rutin and quercetin as indicated by decrease in the number of wriths. Onset of convulsions induced by Pentylenetetrazol was also delayed in the test groups receiving flavanoids. The antistress effects were more prominent at higher doses i.e. 100 mg/kg for both rutin and quercetin. The findings of the present investigations indicate that the isolated compounds rutin and quercetin has significant antistress activity. Conclusion: This is the first report of antistress activity of rutin and quercetin isolated from Ficus benghalensis leaves. The results also revealed that flavonoids (rutin and quercetin) are novel compound for the treatment of neurobiological disorder (stress). Recent investigation of traditional herbal remedy (rutin and quercetin) in controlling and treating diseases tend to prefer natural rather than synthetic ones.
1. INTRODUCTION

Stress is “a condition of psychological and physiological imbalance resulting from exceeded demand and person’s ability to meet those needs”. Stressful circumstances are faced by every human in our day-to-day life and it is characterized by physiologic, behavioral, neuroendocrine as well as emotional responses to threatening stimuli. Stress disturbs the body’s normal homeostasis, also over stress affects cognitive functions and contributes to the development of disorders such as depression, anxiety, Alzheimer’s disease, and Parkinson’s disease. World health organization defined stress as “The pattern of physiological reaction that prepares an organism for action”. It varies from person to person. When the hypothalamus senses stress, chain of reactions is initiated which produces general adaptation syndrome and the stimuli that produce the syndrome are called stressors. Stress is a potent contributor to psychosocial and physical pathological conditions in humans. For the relief and prevention of stress, wide variety of medications can be used. There is no one specific family of medicines that is used to decrease stress. Range of medications may be prescribed for stress-related symptoms.

Many of the useful medications to relieve stress such as- Sedative (CNS depressant) medications, beta-Blockers are also addictive. Using such substances may possess serious behavioral and health problems unless care is exercised. In spite of the great advances in modern medicine, plants still make an important contribution to health care. Pharmaceutical companies are showing renewed interest in investigating higher plants as sources for new lead structures and also for the development of efficacious and safe phytomedicines. The ecosystem of Ficus species is one of the important ecosystems and has great economic and medicinal values. Ficus benghalensis (FB) commonly known as Vad in Marathi is a rich source of medicinal value having multidimensional curative properties. Different parts of the tree have been found to possess medicinal properties; leaves are used for treating ulcers, aerial roots for gonorrhea whereas seeds and fruits are cooling and tonic. The roots of F. benghalensis are given for obstinate vomiting and infusion of its bark is considered as a tonic and is also used in dysentery, diarrhea and diabetes. In India, milky juice (latex) of stem bark of F. benghalensis is used for the treatment of rheumatism and other inflammatory diseases. Photochemical investigation of F. benghalensis explored wide variety of constituents which are responsible for its wide range of pharmacological activities. They include...
ketones, flavonoids, flavonols, sterols, pentacyclic triterpenes and triterpenoids, furocoumarin, tiglic acid ester and some other estersiv.

![Fig 1: Ficus benghalensis](image)

Flavanoids have been acknowledged for their interesting medicinal propertiesv. An important group of naturally occurring, bioactive polyphenolics, ubiquitous in plants of higher generation is present in flavonoidsvi. Flavonoids have potential positive effectvii. Natural flavones and their synthetic derivatives have demonstrated numerous biological activities, including antioxidant, anti-inflammatory, antitumor, anti-allergic, neuro-protective, cardioprotective and antimicrobial. The antioxidant properties of flavones allow them to demonstrate potential application as protective and attenuating agents in oxidative stress. Hence, the present work was undertaken to inspect the anti-stress potential of isolated flavonoids, rutin and quercetin from the leaves of *F. benghalensis*viii.

2. MATERIALS AND METHODS

2.1 Collection of the plant

Fresh leaves of *Ficus benghalensis*, commonly known as vad, were collected in July 2014 from local area, Navi Mumbai, Maharashtra and authenticated by Dr. Rajendra D. Shinde, Botanist, Associate Professor at St. Xavier’s College, Mumbai-400001. The coarse powder of dried leaves of *Ficus benghalensis* was extracted with 70% methanol in Soxhlet extractor. Further, these extracts were subjected to a battery of phytochemical tests to evaluate the constituentsix.
2.2 Isolation of flavonoids

The air-dried powdered leaves of *Ficus benghalensis* (2.5 kg) were extracted with 70% methanol at room temperature. The defatted methanolic extract was successively extracted with chloroform, ethyl acetate, and n-butanol. Further chromatographic isolation of ethyl acetate fraction gives the required flavanoids. The obtained compounds were confirmed by performing Thin layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR), UV spectrophotometry and High performance liquid chromatography (HPLC).

2.3 Animals

Male Swiss albino mice, weighing 15-25 gm, were used. The animals were procured from Bharat serums and vaccines limited, Thane, India. All experimental procedures were carried out in strict accordance with the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA) and were approved by the Institutional Animal Ethics Committee (IAEC). {Protocol number: OCP/IAEC//2014-15/03}

2.4 Drugs

Diazepam (Zepose® 5 mg, Cipla Limited, and Mumbai) at dose of 2 mg/kg was used. Pentylenetetrazol (PTZ) was obtained from (S.D. Fine Chemicals, Mumbai, India). Standard rutin and quercetin were obtained from Yucca Enterprises Pvt. Ltd. All the chemicals and reagents used for the biochemical studies were commercial analytical grade.

2.5 Evaluation of *in vivo* anti-stress activity of rutin and quercetin.

The mice were randomly divided into six groups of six animals each for every model. The treatment groups were pretreated with rutin (80 mg/kg, 100 mg/kg, p.o.) and quercetin (80 mg/kg, 100 mg/kg, p.o.). The control group was pretreated with normal saline (10 ml/kg, p.o.) while the positive control group received diazepam (2 mg/kg, i.p.). Dosing was done for 7 days.
2.5.1 Forced swimming test (FST)

FST was carried out after one hour of oral administration of the flavonoids and after 30 minutes of intraperitoneal administration of the diazepam, using a polypropylene vessel containing water up to 15 cm, and the immobility time was recorded for 6 minutes\textsuperscript{ii}.

2.5.2 Tail Suspension Test (TST)

Animals were suspended individually by end of tail with adhesive tape with the head 50 cm from the bottom. Mice were suspended for a total of 6 min. During the final 4 min interval of the test, duration of immobility was recorded. Mice were considered immobile only when they hung passively and completely motionless\textsuperscript{x}.

2.5.3 Elevated plus maze model

After 30 minutes of dosing the animals were placed individually in the center of the maze and following parameters were noted for 5 minutes; first preference of mouse to open or enclosed arm, number of entries in open and enclosed arm and average time each animal spends in each arm. Performance of animal to open/enclosed arm, average time spent in open arm and number of entries in open arm in each group was compared\textsuperscript{xi}.

2.5.4 Anoxic tolerance test

The mice were subjected to anoxic stress by keeping them in a confined airtight 250 ml glass jar. The time taken for the mice to exhibit the first clonic convulsion was taken as the end point. The animals were removed immediately from the vessel for recovery and resuscitated if needed\textsuperscript{ii}.

2.5.5 Pentylenetetrazol (PTZ) induced convulsion

First group was used to study the effect of PTZ, second group to study the protective effect of diazepam and other 4 test groups for test drug. PTZ was injected to control animals and the onset of action and severity of convulsions were noted. PTZ was injected after 30 min to the animals which have received diazepam and test drugs. Either delay or complete abolition of convulsions in mice treated with diazepam and test drug was noted\textsuperscript{ixi}.
2.5.6 Writhing test

(1% v/v) acetic acid solution was administered to the first group which serves as control and mice were observed. To the second group of animal diazepam was injected and to the test groups (i.e. 4 test groups) test drug was administered, 15 min later, acetic acid solution (1% v/v) was administered to these animals. Onset of wriths, number of abdominal contractions, trunk twist response and extension of hind limbs and also the number of animals showing such response was recorded during period of 10 min. Mean writhing scores in control and test drug-treated groups was calculated

3 RESULTS

3.1 Identification of isolated rutin and quercetin by Spectrophotometric analysis, thin layer chromatography, FTIR and HPLC method.

Rf values of standard rutin and isolated rutin & standard quercetin and isolated quercetin.

Table 1:

<table>
<thead>
<tr>
<th>Solvent systems in TLC and paper chromatography</th>
<th>Rf value of isolated rutin</th>
<th>Rf value of standard rutin</th>
<th>Rf value of isolated quercetin</th>
<th>Rf value of standard quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate: formic acid: acetic acid: water</td>
<td>0.38</td>
<td>0.35</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>Acetic acid: water</td>
<td>0.57</td>
<td>0.60</td>
<td>0.32</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Fig 2: TLC of std and isolated quercetin
Fig 3: TLC of std and isolated rutin

UV spectra of isolated and standard quercetin:

Fig 4: UV spectra of standard quercetin

Fig 5: UV spectra of isolated quercetin.

UV spectra of isolated and standard rutin:

Fig 6: UV spectra of standard rutin.

Fig 7: UV spectra of isolated Rutin.

Fourier transform infrared spectroscopy (FTIR) of std quercetin, rutin and test samples.

Fig 8: FTIR spectra of standard rutin.

Fig 9: FTIR spectra of isolated rutin.
Fig 10: FTIR spectra of standard quercetin.  Fig 11: FTIR spectra of isolated quercetin.

- HPLC performed on standard and isolated quercetin showed that both std and isolated quercetin was eluted at retention time of 3.4 minutes.

Fig 12: chromatogram of standard quercetin  Fig 13: chromatogram of isolated quercetin

- HPLC performed on standard and isolated rutin showed that both std and isolated rutin was eluted at retention time of 2.8 minutes.

Fig 14: chromatogram of standard rutin  Fig 15: chromatogram of isolated rutin.
3.2 Forced swimming test

There was marked increase in swimming time observed with both flavonoids treated animals. Rutin and quercetin caused a significant (P<0.001) anti-stress effect after oral administration at 80 mg and 100 mg/kg respectively. It has to be noted that both the flavonoids treated mice significantly (P< 0.001) decreases the immobility time when compared to standard group and control group [Fig16].

3.3 Tail Suspension Test (TST)

Quercetin at its highest dose of 100 mg/kg dose did not cause an appreciable decrease in immobility time when compared to highest dose of rutin treated mice. Rutin and quercetin at the dose of 80 mg/kg appreciably decreased immobility time indicating its significant (P<0.01) antistress effect when compared to control. [Fig17]

3.4 Elevated plus maze model:

The result showed that the number of open arm entries and time spent in the open arms were increased significantly (P<0.001) after treatment with Rutin at 80 mg/kg and 100 mg/kg respectively whereas quercetin at the dose of 80 mg/kg and 100 mg/kg showed good significant (p<0.01) activity [Fig18].

3.5 Anoxic tolerance test:

In this study, both the flavonoids have significantly enhanced anoxia stress tolerance time evidenced by delaying convulsive signs. Rutin has produced better effect than quercetin. After treatment with Rutin at 80 mg/kg and 100 mg/kg the mean duration of tolerance significantly increases (P<0.001) dose-dependently when compared with quercetin treated mice and control group [Fig 19].

3.6 Pentylenetetrazol induced convulsion:

In the present study, onset of jerk and onset of clonus was increased significantly (p<0.001) when treated with both doses of rutin at 80 mg/kg and 100 mg/kg when compared with the control group. Whereas quercetin at its highest dose (100 mg/kg) showed significant (p<0.001)
increase in the onset of jerk when compared to control group. Also, duration of clonus decreased significantly (p<0.001) when treated with both the doses of rutin at 80 mg/kg and 100 mg/kg when compared with the control group and quercetin at its highest dose (100 mg/kg) showed significant (p<0.001) decrease in the duration of clonus when compared to control group [Fig20].

3.7 Writhing Test:

Rutin and quercetin showed a significant (p<0.001) reduction in no. of wriths within 10 min in acetic acid-induced stress mice when compared to stress untreated mice. Rutin at the dose of 80 mg/kg and 100 mg/kg was more effective than quercetin at the same dose of 80 mg/kg and 100 mg/kg in reducing the no. of wriths in acetic acid-induced stress in mice [Fig 21 and 22].

4. Statistical analysis

Expressed as mean ± S.D. (n = 6), one way ANOVA followed by Dunnett test;
* P < 0.05, ** P < 0.001 when compared with the control group.

**Fig 16:** Effect of rutin and quercetin on immobility time in the Forced Swim test;

**Fig 17:** Effect of rutin and quercetin on Immobility time in Tail Suspension test;
Fig 18: Effect of rutin and quercetin on the various parameters evaluated in Elevated Plus Maze Model;

Fig 19: Effect of Rutin and Quercetin on Anoxia stress tolerance test;

Figure 20: Effect of Rutin and Quercetin on onset of jerks, clonus and duration of clonus in PTZ induced convulsion test

Figure 21: Effect of Rutin and Quercetin on the number of writhing in the writhing test

Fig 22: Percent inhibition of no. of wriths in writhing test by rutin and quercetin.
5. DISCUSSION

Modern lifestyle has increased the exposure of human beings to stressful conditions resulting in the physical plus psychological abnormalities. Therefore, enhancing the adaptability of human beings to stressful conditions is needed. Few synthetic drugs are available, but due to high cost and side effects associated with them. Researchers are looking for alternative methods like Yoga and herbal medicines. Since the introduction of adaptogens, several plants that had once been used as tonics have been investigated in Ayurvedic medicine for their adaptogenic and rejuvenating properties. Flavonoids may lend a hand to provide protection against these diseases by contributing along with antioxidants vitamins and enzymes. By performing TLC, FTIR, UV and HPLC analysis, it was concluded that the isolated flavonoids are rutin and quercetin. In the present study, the antistress activity of rutin and quercetin (80 mg/kg, 100 mg/kg) has been evaluated using various acute stress experimental models. Increase in plasma levels of adrenaline and noradrenaline during stress induced by swimming endurance test has been reported. Results of the swimming endurance test indicate clearly that rutin (80 mg/kg, 100 mg/kg) and quercetin (80 mg/kg, 100 mg/kg) respectively have the properties, whereby, they increase the physical endurance and overall performance in mice. Normalizing the plasma level of catecholamine and monoamine oxidase may be the possible mechanism of action. The Tail Suspension Test shows a strong sensitivity to monoamine. In this study, the flavonoids rutin and quercetin significantly reduced the immobility time by 58.67 sec and 127.17 sec respectively at highest dose of 100 mg/kg which was comparable to that of standard drug diazepam. Thus, the flavonoids rutin and quercetin showed antistress activity probably by inhibiting Monoamine Oxidase-A and Monoamine Oxidase-B, thus increasing the levels of monoamines. One of the experimental models used in the evaluation of anti-anxiety activity is elevated plus maze. Animals tend to avoid open arm entries and prefer to stay in the closed arm due to fear when they are exposed to the new environment. After administration of rutin and quercetin significantly increased the time spent in open arm and lessen the time spent in the closed arm compared to the control group indicating that test drugs could reduce the fear and anxiety in mice. Hence, it can be proposed that the underlying mechanism for antistress activity of flavonoids is analogous to standard drug diazepam. Brain neurotransmitters, i.e. norepinephrine, dopamine, serotonin and acetylcholine are decreased if the mice are exposed to hypobaric environment for a specified period. And also, all the body functions, including

cellular respiration depends on oxygen supply. Increased adaptation due to the depletion of any vital elements during stress by any drug that increases the tolerance can act as adaptogenic agent^{xxi}. In the present study depletion of oxygen in airtight vessel causes convulsions in animals and pretreatment with test doses of rutin and quercetin had increased stress tolerance indicating their anti-stress activity. Both doses of rutin (80 mg/kg and 100 mg/kg) have produced better effect than quercetin (80 mg/kg and 100 mg/kg) respectively. Hence, two possible mechanisms of flavonoids can be proposed. The significant increase in anoxia tolerance time is an indication of either resistance to it or reduction in cerebral oxygen consumption or^{xiii} this may be due to flavonoids were capable of increasing the enzyme succinate dehydrogenase in the brain which is responsible for consumption and maintenance of energy in the cellular system of the organism, which helps in adaptive processes during stress. Gamma amino butyric acid which produces a marked inhibitory effect on hypothalamic-pituitary-adrenal (HPA) axis activity is also decreased during stress^{xvi}. PTZ, a GABA receptor antagonist, is a well-established animal model to evaluate efficacy of drugs against generalized absence seizures in humans^{ii}. PTZ is known to block the action of GABA in the CNS, inducing convulsion. The antistress activity of rutin and quercetin against PTZ is observed by increasing the latency of the onset of convulsions, mortality protection, and decreasing the duration of convulsions. Administration of rutin and quercetin affects seizures induced by PTZ. It may be possible that rutin and quercetin modulate Gamma amino butyric acid receptors and also acts as N-Methyl D-Aspartate receptor antagonist^{xxii}. In acetic acid induced writhing test, the administration of acetic acid caused hyperalgesic effects on the pain pathway which results in an increase in the number of writhes indicating stress development^{xxiii}. There is an increase in level of prostaglandins in peritoneal fluids following administration of acetic acid^{xxiv}. Prostaglandins are involved in the regulation of HPA axis activity under basal and stress conditions by neurotransmitters and neuropeptides^{xxv}. The results of acetic acid-induced writhing test in the present study show the decrease in the number of writhes with the treatment which indicates clearly that all the doses (80 mg/kg and 100 mg/kg) of both the flavonoids rutin and quercetin respectively can play a significant role in the inhibition of pain processes, which shows that the extracts have anti-stress property. The antistress activity of plant may be attributed to the inhibition of prostaglandin synthesis^{xxvi}. 
6. CONCLUSION

The present research was carried out to evaluate phytopharmacological potential of the plant *Ficus benghalensis* using *in-vivo* pharmacological screening models. The isolated flavonoids were identified by thin layer chromatography and spectrophotometric analysis viz. HPLC, UV and FTIR. In conclusion, our results provide evidence that the seven-day treatment with the isolated flavonoids rutin and quercetin show antistress (adaptogenic) activity in various acute and sub-acute stress models. In FST and TST, there was strike decrease in immobility time in the stressed mice. Time spent in open arm was gradually increased after the 7 days treatment with the rutin and quercetin in elevated plus maze model. In anoxia test, there was an enhancement of duration of tolerance which showed the good antistress activity. The antistress activity of rutin and quercetin against PTZ is observed by increasing the latency of the onset of convulsions, mortality protection, and decreasing the duration of convulsions. Numbers of wriths were considerably reduced in acetic acid induced writhing test. The observed antistress activity may be due to the prevention of desensitization of both the and central components of the HPA and due to the non-specifically increased resistance produced by the rutin and quercetin. This study provides significant evidence of the medicinal and traditional uses of flavonoids from *Ficus benghalensis* in stress disorders.

AUTHORS’ CONTRIBUTIONS

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

Authors have declared that no conflict of interests exist.

REFERENCES


Veeresh BP. A study on adaptogenic activity of tuber extract of Pueraria tuberose, Rajiv Gandhi University of Health Sciences, Karnataka, Bangalore, 2005.


Robert b. Chapter 2

George E. Physiology of Stress, john and barlett publishers

Patel et al., in vitro free radical scavenging activity of ficus benghalensis lin and ficus racemosa linn. leaf extracts, pharmacology online 2010;1: 950-957

Mohamed ES et al. bio-guided isolation and structure elucidation of antioxidant compounds from the leaves of ficus sycomorus, pharmacology online 2010; 3: 317-332


Tâmara CD, Juliane CS et al., The Role of Flavonoids on Oxidative Stress in Epilepsy, Oxid Med Cell Longev Volume 2015, 9 pages


