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Anti-stress Activity of a Bioflavanoid: Quercetin from *Euphorbia hirta*

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Authors' contributions

This work was carried out in collaboration between all authors. Author NT designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author AM managed the literature searches, analyses of the study performed the spectroscopy analysis and author GB managed the experimental process and author AC procured the plant. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was undertaken to investigate the antistress potential of isolated compound, Quercetin from *Euphorbia hirta*.
Study Design: In the present study extracted leaves of *Euphorbia hirta* were subjected to isolation and purification of phytoconstituent as marker. The structure of isolated compound was characterized and elucidated with chemical and spectroscopic techniques such as Infra Red, Nuclear Mass Resonance and Mass spectroscopy experiments. The anti-stress activity of isolated compound i.e. Quercetin from *Euphorbia hirta* using elevated plus maze (EPM) model and forced swimming test (FST) respectively in swiss albino mice.
Methodology: The hydroalcoholic extract of *Euphorbia hirta* was partioned successively with petroleum ether, ethyl ether and ethyl acetate using separating funnel. Ethyl acetate fractions were subjected to silica gel column chromatography for the isolation of Quercetin. The structure was elucidated by spectroscopic methods. Albino mice were treated with different doses of the isolated compound Quercetin (i.e.25, 50 and 100 mg/kg orally) and behavior was observed on the EPM and FST.

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Results: The crude hydroalcoholic extract of *Euphorbia hirta* was successively partitioned with various solvents. Chemical structure of Quercetin was elucidated by IR, NMR and MASS spectroscopy. Mice pretreated with Quercetin at the dose of 25, 50 and 100 mg/kg show significant improvement in the swimming time. On the other hand, after administering 25, 50 and 100 mg/kg Quercetin significantly increased the time spent in open arm and decreased the time spent in the closed arm compared to the control group The anti stress effect of the Quercetin was prominent at 100 mg/kg. The result of EPM model and FST revealed that isolated compound having antistress activity.

Conclusion: This is the first report of antistress activity of Quercetin and isolation of Quercetin by column chromatography from hydroalcoholic exract. The results also revealed that Quercetin is a novel compound for the treatment of neurobiological disorder (stress). Recent investigation of traditional herbal remedy (Quercetin) in controlling and treating diseases tend to prefer natural rather than synthetic ones.

Keywords: Traditional herbal remedies; Spectroscopic authentication; Elevated plus maze; Forced swimming test; Anti- stress therapeutics.

1. INTRODUCTION

The human society has become complex and in many ways, more demanding. However, our physiological responses designed to cope with the ever-increasing adverse situations have not evolved appreciably during the past thousand years. Adaptability is probably the most distinct characteristic of life. Dr. Hans Seyle defined stress as the sum of all non-specific responses of the body to any external stimuli acting upon it. Fundamentally, it is a physiological response towards external stimuli and the primary objective of such a response is to restore the normal process of life. Perhaps the single most important property of an adaptogen is its proven ability to combat stress in all forms [1]. Stress is increasingly recognized as the precipitant of several psychiatric illnesses, including anxiety and depression [2]. World Health Organization estimates that 80% of the world's population depending on traditional medicine for their health needs. In many developed countries, traditional herbal remedies are making a comeback as alternatives to modern medicine. The existence of traditional medicine depends on plant diversity and the related knowledge of their use as herbal medicine [3]. Therefore, herbal therapies should be considered as alternative/complementary medicines. Recently, the search for novel pharmacotherapy from medicinal plants for psychiatric illnesses has progressed significantly. This has been reflected in the large number of herbal medicines whose psychotherapeutic potential has been assessed in a variety of animal models [4]. There are a number of recent publications concerning the importance of fruit and vegetable diet in the prevention of many diseases. The concept of food *synergy* becomes particularly important in view of the lack of effect of many isolated compounds shown in clinical trials. The acceptance of this concept has the potential to reduce the costs associated with an over dependence on pharmacological approaches to disease management. Another example of the beneficial effect of natural food products is isoprenoids, found in many fruits and plants including citrus (perillylalcohol, geraniol), sage, spearmint, nutmeg (perillyl alcohol), basil (geraniol), lemon grass (farnesol and geraniol), and chamomile (farnesol) that are essential in the regulation of cell proliferation, apoptosis, differentiation, and lipid biosynthesis [5].

Flavonoids, a group of secondary metabolites widely distributed in the plant kingdom, have been acknowledged for their interesting medicinal properties [6]. Flavonoids comprise an important group of naturally occurring, bioactive polyphenolics, ubiquitous in plants of higher generation [7]. Flavonoids and food products containing them have potential positive effect [5]. Among them, natural flavones, as well as some of their synthetic derivatives, have been shown to exhibit several biological activities, including antioxidant, anti-inflammatory, antitumor, anti-allergic, neuroprotective, cardioprotective and antimicrobial. The antioxidant properties of flavones allow them to demonstrate potential application as preventive and attenuating agents in oxidative stress [6]. Among flavonoids, the flavonol quercetin is one of the most widely distributed in human dietary sources [8].

In addition, *Euphorbia hirta* is popularly known as *Euphorbia pilulifera*. It is commonly called Australian asthma herb [9]. The leaves of *E. hirta* are found to contain flavonoids, polyphenols,

tannins, sterols, alkaloids, glycosides and triterpenoides [10].

Anxiolytic property of *E. hirta* was demonstrated in chronically stressed rats subjected to EPM. *E. hirta* treatment showed marked anti-anxiety activity in CIS rats. Co-treatment of rats with flumazenil, bicuculline or picrotoxin resulted in a significant reduction of anxiolytic effect of *E. hirta* indicating that its actions are mediated through GABAA receptor-benzodiazepine receptor-Cl channel complex. Acetylcholine and the cholinergic system are also known to involve in anxiety. Further study showed that anxiolytic effects of *E. hirta* in rats subjected to CIS was due to suppression of CIS-induced AChE activity in the frontal cortex, hippocampus, and septum brain regions [2]. It is also used as antidiarrheal, antispasmodic, anti-inflammatory, antifungal, anticancer, antimalarial, antiamoebic, antibacterial and antihelmentic etc [10].

Quercetin is a unique bioflavonoid that has been extensively studied by researchers over the past 30 years [11]. It has been reported that Quercetin possesses many biological effects, including antidepressant [12], cardioprotective [13], anticancer [14], gastroprotective [15], antitumor [7] and antimicrobial [16] effects. Furthermore, strong antioxidative effect of Quercetin has also been documented [17]. Quercetin has also been demonstrated to display the antiviral and antiinflammatory effects [18]. Scientific study has established the multifarious utility of this plant in wide array of pharmacological activities. However, there is no report available on anti stress activity of the Quercetin from the leaves of *Euphorbia hirta*. Hence the present work was undertaken to investigate the antistress potential of isolated compound, Quercetin from *Euphorbia hirta*.

The present manuscript revises relevant studies focusing the preventive effects of flavones like Quercetin from *Euphorbia hirta* on stress-related diseases, namely the neurological diseases and its associated complications. This study also revealed the broader objective of providing a cheap and safe remedy for human health problems.

2. MATERIALS AND METHODS

2.1 Plant Material

The leaves of *Euphorbia hirta* were collected from the High Altitude Plant Physiology Research Centre (HAPPRC), Srinagar, Uttarakhand, India in the month of march 2011 and deposited in National Botanical Research Institute, Lucknow, India for taxonomical authentication. The leaves were air dried for 20 days and crushed into a coarse powder with a grinder and passed through 40-mesh sieve. They were stored in a well closed container separately.

2.2 Extraction and Isolation of Compound from Leaves of *Euphorbia hirta*

The hydroalcoholic extract was re-extracted successively with petroleum ether (fraction I), ethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, whereas ethyl acetate fraction was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h and filtered. The filtrate was extracted with ethyl acetate and washed with distilled water to neutrality. Ethyl acetate fractions were subjected to silica gel (60-120 mesh) column chromatography for the isolation of individual phytoconstituent. Ethyl acetate soluble part was eluted gradiently using chloroform, chloroform: ethyl acetate (50:50), ethyl acetate, ethyl acetate: methanol (90:10) and methanol to give a compound which shown a single spot on the TLC plate developed in Toluene: ethyl acetate: formic acid (6:2:0.8) and sprayed with Aluminium chloride reagent. This isolated compound further confirmed by using UV and FTIR spectroscopy.

2.3 Spectroscopic Authentication

Spectral analysis FTIR, ^{1}H NMR and Mass of Isolated compound Quercetin was performed at Sophisticated Analytical Instrument Facility, Central Drug Research Institute, Lucknow, India to authenticate the functional group, molecular weight and molecular formula. FTIR spectra were recorded on Perkin Elmer Spectrum RX1 using alcohol. ^{1}H NMR (400 MH_z) spectra were recorded on Bruker Advance 400 in $CDCl_3$ with tetramethylsilane as internal standard. The FAB mass spectra were recorded on a Jeol SX102/Da-600 mass spectrophotometer/Data System uses Argon/Xenon 6 Kiev, 10 mA0 as the FAB gas. The accelerating voltage was 10 KV.

2.4 Experimental Animals

Albino mice (20-25 g) were bought from the Animal House of Siddhartha Institute of pharmacy, Dehradun, Uttarakhand, India. The animal room was maintained on a 12-h light and dark cycle with a constant temperature and humidity. Standard pellet food and tap water is available ad libitum. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Department of Pharmacology of the Siddhartha Institute of Pharmacy, Dehradun, Uttarakhand, India. The experiments were conducted in a sound proof laboratory.

2.5 Experimental Design

The experimental animals were divided into five groups. The group I was control and was given normal saline in a dose of 10 ml/kg, p.o. Group II was a positive control and was given standard drug, Diazepam (2 mg/kg, i.p.), suspended in the vehicle. Group III-V was treated as test groups and was given isolated Quercetin at different doses, i.e. 25, 50 and 100 respectively. All the test solutions, standard drug and control were administered orally 30 minutes prior to the experiment.

2.6 Assessment of Antistress activity

2.6.1 Elevated plus maze test

Antistress activity was evaluated using the elevated plus maze model. The studies were carried out on mice. The plus-maze apparatus was made of Plexiglas and consisted of two open (30X5 cm) and two enclosed (30X5X15 cm) arms. The arms extended from a central platform of 5X5 cm. The apparatus was mounted on a Plexiglas base, raising it 38.5 cm above the floor and illuminated by red light. Each mouse was placed individually at the center of elevated plus maze with its head facing toward an open arm and observed for 5 min to record the time spent in each arm [19].

2.6.2 Forced swimming test

The forced swim test was carried out on mice individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 15 cm of water at 25±1 °C; the total duration of immobility during the 6-min test was scored. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. The duration of immobility was recorded [20].

2.7 Statistical Analysis

All data are expressed as the mean ± S.E.M and were obtained from four distinct experiments. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dunnett's test. The significant difference was set at $p < 0.05$

3. RESULTS

3.1 Structure Elucidation of Isolated Compound Quercetin from Leaves of *Euphorbia hirta*

FTIR spectra showed a medium peak of the functional groups OH at 3343 cm^{-1}, CC aliphatic (2921 cm^{-1}), the strong peak of ether C-O stretching was observed at 1161 cm^{-1}. Medium and weak peaks were observed at 1450 cm^{-1} and 1381 cm^{-1} indicating C=C stretching of aromatic ring. Sharp peaks at 1715 cm^{-1} indicating C = O group.

Identification by 1H NMR spectra showed the presence of five aromatic protons, hydroxyl proton, five aliphatic protons and 3 methyl protons.

Mass spectroscopy showed the molecular ion at m/z 302 (M^++1) corresponding to molecular formula ($C_{15}H_{10}O_7$) 301, as supported by ^{1}H NMR, on the basis of their analysis.

From the spectral data the structure of the compound EH was elucidated as Quercetin with molecular formula $C_{15}H_{10}O_7$.

3.2 Elevated Plus Maze Test

It was found that there is a significant increase in the time spent in the open arm (Fig. 1) in mice treated with diazepam 2 mg/kg (345.8±1.83 Sec) ($p<0.05$) and 25, 50 and 100 mg/kg of Quercetin (127.0±1.71, 147.2±2.18 and161.5±1.33 Sec) ($p<0.05$) when compared to control (80.1±3.32 Sec). It was also found that there is a significant decrease in time spent in the closed arm (Fig. 2) in mice treated with diazepam 2 mg/kg (510.6±1.65) ($p<0.05$), Quercetin at 25 mg/kg

(593.2±6.56), 50 mg/kg (560.8±4.23) and 100 mg/kg (542.0±2.20) ($p<0.05$) respectively when compared to control (609.2±4.75).

3.3 Forced Swimming Test

The control animals remained immobile for most of the time during the test session and immobility time of control group was ($p < 0.05$) 187.1 ± 0.85 second. Immobility time (Fig. 3) of mice treated with Quercetin was ($p < 0.05$) 194.7 ± 0.69, 247.6 ± 1.97 and 286.9 ± 4.41 second at the dose of 25, 50 and 100 mg/kg orally in a dose-related manner while mice treated with standard diazepam was 516.9± 1.93 second at the dose of 2 mg/kg.

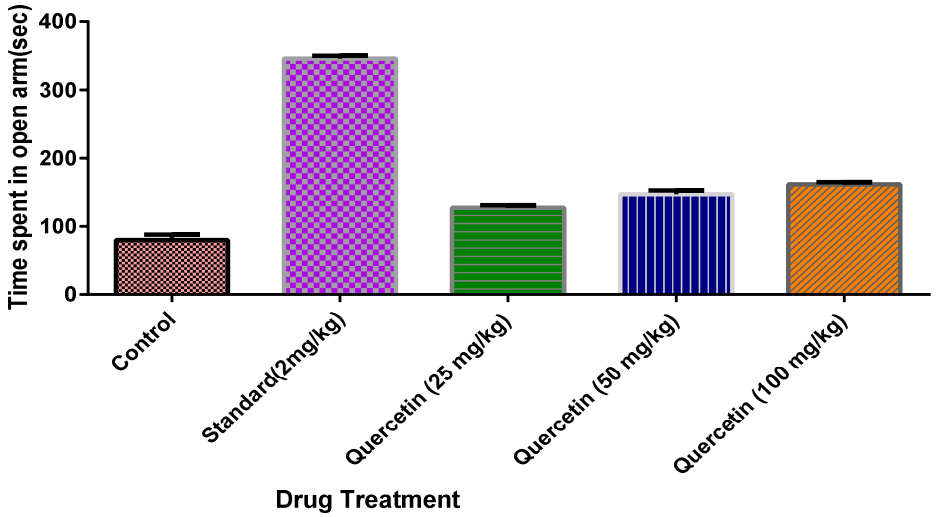

Fig. 1. Time spent (Sec) in open arm of elevated plus maze model (Bar 1: Control; Bar 2: Standard 2mg/kg; Bar 3: Quercetin 25mg/kg; Bar 4: Quercetin 50 mg/kg; Bar 5: Quercetin 100mg/kg). Value represent mean ± S.E.M, $p < 0.05$, n=6

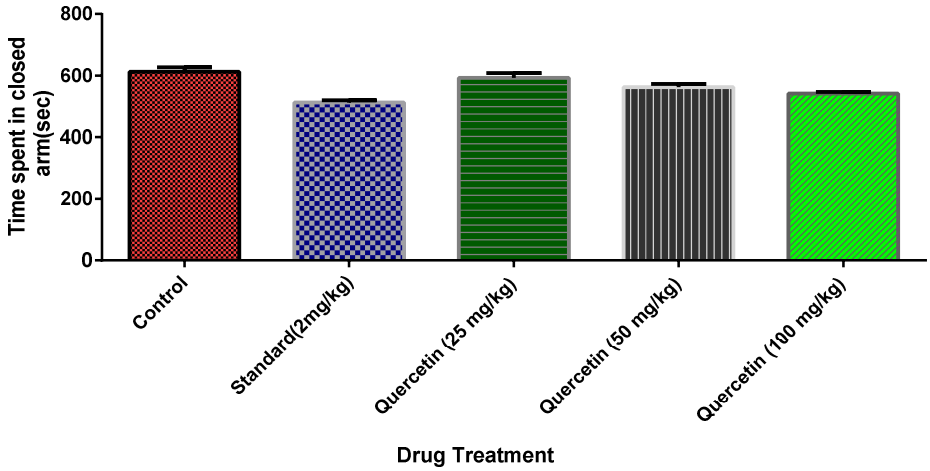

Fig. 2. Time spent (Sec) in close arm of elevated plus maze model (Bar 1: Control; Bar 2: Standard 2mg/kg; Bar 3: Quercetin 25mg/kg; Bar 4: Quercetin 50 mg/kg; Bar 5: Quercetin 100mg/kg). Value represents mean ± S.E.M, $p < 0.05$, n=6

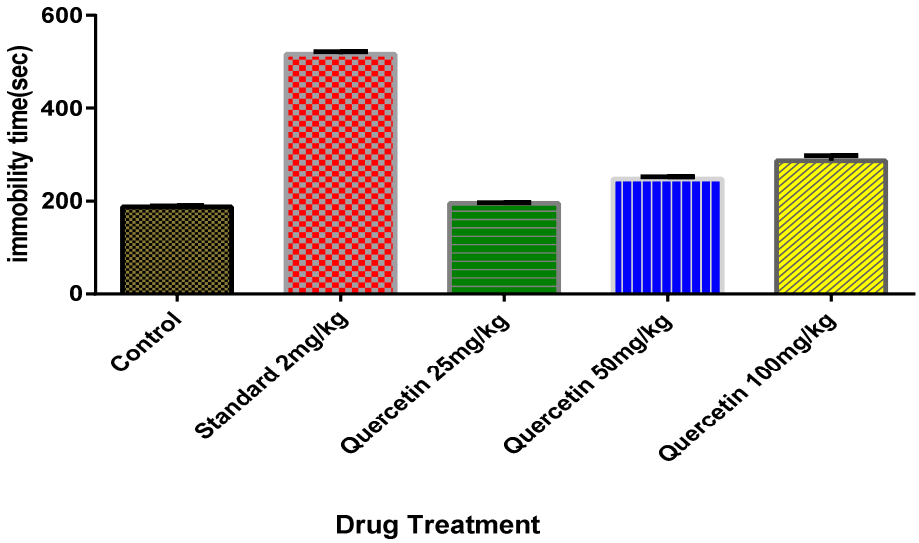

Fig. 3. Immobility time (Sec) in forced swimming test (Bar 1: Control; Bar 2: Standard 2mg/kg; Bar 3: Quercetin 25mg/kg; Bar 4: Quercetin 50 mg/kg; Bar 5: Quercetin 100mg/kg). Value represents mean ± S.E.M, $p < 0.05$, n=6

4. DISCUSSION

Modern lifestyle has enhanced the exposure of human beings to stressful conditions resulting in the physical, psychological abnormalities. Therefore, there is a need to enhance the adaptability of human beings to stressful conditions. Few synthetic drugs are available, but due the cost and the side effects associated with them, the researchers looking for alternative methods like Yoga, Herbal medicines [21]. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. Flavonoids may help provide protection against these diseases by contributing along with antioxidants vitamins and enzymes .Quercetin is a type of flavonol [22]. In the present study an attempt was made to evaluate the antistress property of traditionally used plant by name *Euphorbia hirta*. Quercetin is the major chemical constituent of the *Euphorbia hirta*. It acts on various multiple pharmacological mechanisms [11]. Therefore, hydoalcoholic extract contains bioactive entities with potential antitstress activity. In this study column chromatography separation, and by the TLC purification of fractions from hydoalcoholic extract have lead the isolation Quercetin. Spectroscopic results (FTIR, ^{1}H NMR and MASS) confirm that isolated compound is Quercetin. To our knowledge compound Quercetin was isolated for the first time from the hydoalcoholic extract of the leaves of *Euphorbia hirta*. Elevated plus maze model and swimming endurance test were used for evaluation of antistress activity in the present study [21].

Mice when forced to swim in a restricted space from which they cannot escape, become immobile after an initial period of vigorous activity, indicating the stress. Mice pretreated with Quercetin show significant improvement in the swimming time [23]. It is well known that drugs with anti-stress properties reduce the duration of immobility in animals [24]. The pretreatment with Quercetin increase swimming endurance in mice. Mice pretreated with Quercetin at the dose of 25, 50 and 100 mg/kg show significant improvement in the swimming time. The anti stress effect of the Quercetin was prominent at 100mg/kg. In the FST all the doses administered were able to reduce immobility time and simultaneously to enhance swimming.

The elevated plus maze is currently one of the most widely used models of animal anxiety, the test is principally based on the exposure of animal to an elevated maze array evokes an approach-avoidance conflict that is considerably stronger than that evoked by exposure to an open maze array. The animals being exposed to the new environment tend to avoid open entries

and prefer to stay in the closed arm due to fear. As expected standard diazepam significantly increased time spent in open arm [25]. After administration 25,50 and 100 mg/kg Quercetin significantly increased the time spent in open arm and decreased the time spent in the closed arm compared to the control group [18] indicating the test drugs could reduce the fear and anxiety in the mice [26].

5. CONCLUSION

The present study investigated the pultative behavioral effects of Quercetin. In conclusion, although Quercetin has been used as an anti-stress remedy in folk medicine. In the present study, we have demonstrated for the first time isolation from the hydroalcoholic extract of the leaves and identification of active anti-stress principles and antistress property of Quercetin from *Euphorbia hirta.* The results also revealed that Quercetin having higher antristress activity. This potent compound from *Euphorbia hirta* may have potential for future development of anti-stress therapeutics.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All the author's hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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