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Evaluation of Anti-Stress Activity of Ethanolic Extract of *Carissa congesta* Wight Leaves in Swiss Albino Mice

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ABSTRACT

Objective: The aim of the study was to evaluate the anti-stress activity of ethanolic extract of *Carissa congesta* Wight leaves in Swiss albino mice. **Methods:** The extract used in this study was prepared by Soxhlet extraction of fresh dried leaves of *Carissa congesta* Wight using ethanol as an extraction solvent. Stress is involved in the pathogenesis of a variety of diseases including hypertension, peptic ulcer, immunodepression, reproductive dysfunction, and behaviour disorder. Overload of stress increases free radicals, produces damage to neuronal receptors and a variety of tissues. In this experimental research study two models, namely, forced swim test (FST) and tail suspension test (TST) were used for the screening of anti-stress activity. 100, 200 and 400 mg/kg ethanolic extracts of *Carissa congesta* Wight were given orally, while 2mg/kg of Diazepam which acts as a standard was given intraperitoneally. The data obtained were analyzed by One-way ANOVA followed by Dunnett Multiple Comparisons test using GraphPad InStat. $p<0.05$ was considered to be significant. **Result:** All three test groups of the ethanolic extract of leaves of *Carissa congesta* Wight showed decrease in immobility time in both FST and TST when compared against control. **Conclusion:** There is substantial evidence that triterpenoids and flavonoids play an active role in providing anti-stress activity. This study is an attempt to find out the alternative medication for treating chronic stress with single medication which was shown a beneficial effect in animal models, may be useful for curing symptoms of stress.

INTRODUCTION

Stress is a common phenomenon that is experienced by every individual. When stress becomes extreme, it is harmful for the body and hence needs to be treated. Stress is involved in the pathogenesis of a variety of diseases including hypertension, peptic ulcer, immunodepression, reproductive dysfunction and behaviour disorder [1]. Stress is simply a reaction to a stimulus that disturbs our physical or mental equilibrium. Acute stress can be exciting; it keeps us active and alert. But chronic stress can have detrimental effects on health [2]. Homeostasis regulates the physiological actions in the body and depends on the stress and antioxidant levels in the cells. Stress is involved in the major portion of alterations of physiological actions, leading to pathogenesis [3]. Overload of stress increases free radicals, produces damage to neuronal receptors and a variety of tissues. Free radical scavenging agents may have a great potential in ameliorating these disease/disorders. Stress basically is a reaction of mind and body against change in the homeostasis. The productive stress is called eustress while harmful stress is called distress. Stress triggers a wide range of body changes called general adaptation syndrome (GAS). The stimuli, which produce GAS, are called stressors and range from physical to psychological factors including cold, heat, infection, toxins, and major personal disappointment [4].

Anti-stress agents decrease stress. Considering the debut of adaptogens, many plants have been explored due to their anti-stress and renovating properties in conventional medicines [5]. Drugs having anti-stress properties induce a state of non-specific resistance against the stressful condition. Drugs like benzodiazepines certain central nervous system (CNS) stimulants such as amphetamines and caffeine as well as some anabolic steroids are routinely used by people to combat stress. The incidence of toxicity and dependence has limited the therapeutic usefulness of these drugs [6]. Alternative is clearly needed because of the inability of the current therapies to manage the condition of disease [7]. The first drug used to treat pathologic condition of the CNS was based on natural resources [8]. Peoples from different areas of world use herbal medicine to alleviate affective disorders [9]. In Mexico, several medicinal plants are used to alleviate insomnia, depressed mood, and anxiety [10]. The herbal formulations claimed to enhance physical endurance; mental functions and non-specific resistance of the body have been termed as adaptogens [11].

Carissa congesta Wight (syn. *C. carandas* Auct. formerly widely shown as *C. carandas* L.) belong to family Apocynaceae. It is called kerenda in Malaya, karaunda in India; Bengal currant or Christ's thorn in South India; namdaeng in Thailand; caramba, caranda, caraunda and perunkila in the Philippines. This species is a rank-growing, straggly, woody, climbing shrub, usually growing to 10 or15 ft (3-5 m) high, sometimes ascending to the tops of tall trees; and rich in white, gummy latex. The leaves are evergreen, opposite, oval or elliptic, 1 to 3 in (2.5-7.5 cm) long; dark-green, leathery, glossy on the upper surface, lighter green and dull on the underside [12]. The leaves of *Carissa congesta* Wight were reported to contain triterpenoid constitutes as well as tannins, and a isomer of urosolic acid namely carissic acid, triterpene carandinol, betulinic acid, β-sitosterol-3-O-β- d-glucopyranoside, oleanolic acid, ursolic acid, and 4-hydroxybenzoic acid [13]. The ethanolic extract of leaf of *Carissa congesta* demonstrated the presence of alkaloids, carbohydrate, unsaturated sterols, phenolics (flavonoids) and saponins [14].

A number of active chemical constituents including phenolic compounds, such as flavonoids, phenolic acids, tannins, lignins, and alkaloids, vitamins serve as useful antioxidants. Antioxidant potential is the best supplements for the diseases associated with oxidative stress [15]. The antistress activity was reported due to the presence of triterpenoid in the fruits of *Carissa carandas* [16]. Hence, an attempt was made to evaluate the antistress activity due to the leaves of *Carissa congesta* as it also contains triterpenoids.

MATERIALS AND METHODS

METHODS

Collection and authentication of plant materials

Fresh leaves of *Carissa spinarum* L. (Synonym: *Carissa congesta* Wight) belonging to family Apocynaceae were collected from Sanjay Gandhi National Park, Borivali East, Mumbai, Maharashtra- 400066 and was authenticated by Dr. Praveen Kale, Botanist at Blatter Herbarium, St. Xavier’s College, Mumbai-400001.

Extraction and phytochemical evaluation

The leaves of *Carissa congesta* Wight were sundried for 7 days and later dried in drier at 40°C for about an hour. The dried leaves were then ground into powder using high capacity grinding machine and stored in air tight plastic container and kept in cool, dark and dry place. The sun dried and powdered plant leaves (500 gm) of *Carissa congesta* Wight was successively extracted in a Soxhlet apparatus at 50°C-60°C temperature using 250 ml of ethanol. All extracts were filtered individually through filter paper and poured on Petri dishes to evaporate the liquid solvents from the extract to get dry extracts. The dry crude extracts were weighed and stored in air-tight container and kept in refrigerator (0-4)°C until use [14]. Suspension of the extract was prepared in 1 % Tween-80 and used to assess pharmacological activities [17]. Extract was tested for the presence of active principles using standard procedures which revealed the presence of triterpenoids, steroids, glycosides, saponins, alkaloids, flavonoids.

Experimental animals

The Institutional animal ethics committee of Oriental College of Pharmacy approved the experimental protocol no. OCP/IAEC/2016-2017/04. The animals used for the experiments were healthy Swiss-Albino mice (either sex) aged 3-5 weeks (20-30g) were procured from Bombay Veterinary college, Mumbai. The animals were group-housed in standard polypropylene cages (6 mice per cage) under good hygienic conditions in the registered animal house and maintained under controlled room temperature (22 +/- 20C) and humidity (55 +/- 5%) with 12-hrs light-dark cycle (lights ON from 7:00 am to 7:00 pm), with food and water available *ad libitum*. All animal experiments were conducted in accordance with the CPCSEA guidelines. Efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Phytochemical analysis [18]

Test for Steroids and Triterpenoids:

Liebermann Burchard test –

Crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids and triterpenoids respectively.

Test for Glycosides:

Keller Killiani Test –

Test extract solution was treated with few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish-brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycosides.

Test for Saponins:

Foam Test –

Test extract solution was mixed with water and shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result.

Test for Alkaloids:

Hager's Test –

Test extract solution was treated with few drops of Hager's reagent (saturated picric acid solution). Formation of yellow precipitate would show a positive result for the presence of alkaloids.

Test for Flavonoids:

Alkaline reagent Test –

Test extract solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow color which would become colorless on addition of few drops of dilute Hydrochloric acid, indicates the presence of flavonoids.

Acute oral toxicity (AOT)

For AOT, the procedure prescribed by the OECD was followed i.e. OECD – 423. Three doses were chosen from the Annex II of OECD 423 i.e. minimum, medium and maximum i.e. 50 mg/kg, 300 mg/kg and 2000 mg/kg after sighting study. A total of 9 animals were chosen i.e. 3 animals per group and three groups were taken namely Group 1 (*Carissa congesta* extract - 50 mg/kg), Group 2 (*Carissa congesta* extract - 300 mg/kg) and Group 3 (*Carissa congesta* extract - 2000 mg/kg). A single dose was administered and animals were observed for a period of 14 days for clinical signs and mortality.

Drugs

Drug extracts (100 mg, 200 mg, and 400 mg)

Diazepam

Distilled water

Dose selection

The extracts were found to be safe at a dose of 2000 mg/kg since no mortality was observed. Doses are selected as shown in Table 1.

Table No. 1: Grouping of animals and dose selection

Treatment	Dose
Normal control (Distilled water)	10 ml/kg (p.o.)
Positive control (Diazepam)	2 mg/kg (i.p.)
Test 1: *Carissa congesta* Wight.	100 mg/kg (p.o.)
Test 2: *Carissa congesta* Wight.	200 mg/kg (p.o.)
Test 3: *Carissa congesta* Wight.	400 mg/kg (p.o.)

SCREENING FOR ANTI-STRESS ACTIVITY

Forced swim test (FST)

A total number of mice were randomly divided into five groups (n=6 per group), namely the normal control (group 1), positive control (group 2) and 3 test groups (100 mg/kg, 200 mg/kg and 400 mg/kg), which were administered 10 ml/kg distilled water, 2 mg/kg body weight diazepam (i.p.) and estimated doses 100, 200 and 400 mg/kg of ethanolic extract of *Carissa congesta* leaves, respectively, *per es* (p.o.) for a total of 7 days. On day 6, all the mice were allowed to swim individually for 6 min for adaptation. On day 7, the mice were allowed to swim individually for 6 min and the duration of immobility (period during which the mice only float in the upright position with minimum movement to keep their heads above water) was scored after placement into the water [19]. The apparatus used in FST was cylindrical glass tank (40 cm height x 25 cm diameter).

Tail suspension test (TST)

After Weighing and numbering the animals were divided into five groups each containing 6 mice. First group was of normal control, second group of positive control and three groups of test substances. Animals were suspended individually by end of tail with Micropore adhesive tape (approximately 1cm) with the head 50 cm from the bottom. Mice were suspended for a total of 6 min. During the final 6 min interval of the test, duration of immobility was recorded. Mice were considered immobile only when they were hung passively and completely motionless [20].

Statistical analysis

All values are expressed as mean±SD. Statistical significance was determined using one-way ANOVA followed by Dunnett Multiple Comparisons Test. $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

RESULTS

Phytochemical analysis

The results of the phytochemical screening of extract of *Carissa congesta* Wight leaves are reported as follows (Table 2).

Table No. 2: Qualitative analysis and phytochemical screening of extract of leaves of *Carissa congesta*

Plant metabolites	Test	Extract of *Carissa congesta* Leaves
Steroids and Triterpenoids	Liebermann Burchard test	+
Glycosides	Keller Killiani Test	+
Saponins	Foam Test	+
Alkaloids	Hager's Test	+
Flavonoids	Alkaline reagent Test	+

AOT study

Acute oral toxicity studies for the test extract were carried out in mice as per the OECD Guideline No. 423. Oral acute toxicity was studied in overnight fasted mice with water. The Mice were randomly allocated into three groups each containing three animals. The observed parameters such as muscular tones, tremors, convulsions, feed and water intake, breathing patterns and presence of mouth secretions were observed for the first 12 hrs and for further 14 days.

The results of these studies are as follows:

Mortality

The extracts were found to be safe at a dose of 2000 mg/kg since no mortality was observed.

Signs and symptoms of toxicity

Signs of intoxication were not observed 24 hours post treatment as dose did not produce any significant changes in behavioral pattern and failed to elicit any clinical abnormality. LD50 was considered as more than 2000 mg/kg.

Results of AOT are shown in Table 3.

Table No. 3: Observation of Acute Oral Toxicity Study [Observed (+); Not observed (–)]

OBSERVATIONAL PARAMETER	**ETHANOLIC EXTRACT OF *Carissa congesta* Wight**
Loss of reflex:	
Righting reflex	-
Pinna reflex	-
Corneal reflex	-
Changes in:	
Body weight	-
Skin	-
Fur	-
Any Clinical Abnormalities:	
Tremors	-
Convulsions	-
Salivation	-
Diarrhoea	-
Lethargy	-
Sleep	-
Coma	-
Death within:	
24 hours	-
1 – 14 days	-

FST

All the three test groups of the ethanolic extract of leaves of *Carissa congesta* Wight showed dose dependent decrease in immobility time when compared against normal control in forced swim test (FST). Results of FST are shown in Table 4 and Fig. 1.

Table No. 4: Effect of extract of leaves of *Carissa congesta* Wight on mobility and immobility time of FST in mice

Treatment	Dose	Immobility time (sec)	Mobility time (sec)
Normal control (distilled water)	10 ml/kg (p.o.)	133.83 ± 2.48	106.17± 2.48
Positive control (Diazepam)	2 mg/kg (i.p.)	59.83 ± 1.94**	180.17± 1.94**
Test 1 *Carissa congesta* Wight.	100 mg/kg (p.o.)	129.33 ± 2.80*	110.67 ± 2.80*
Test 2 *Carissa congesta* Wight.	200 mg/kg (p.o.)	111.17 ±2.31**	128.83 ±2.31**
Test 3 *Carissa congesta* Wight.	400 mg/kg (p.o.)	80.5 ± 2.88**	159.5 ±2.88**

(Each value is given as Mean ± S.D. (n=6) one way ANOVA followed by Dunnett Multiple Comparisons Test; **p<0.01, *p<0.05 when compared to the corresponding values of the normal control**)**

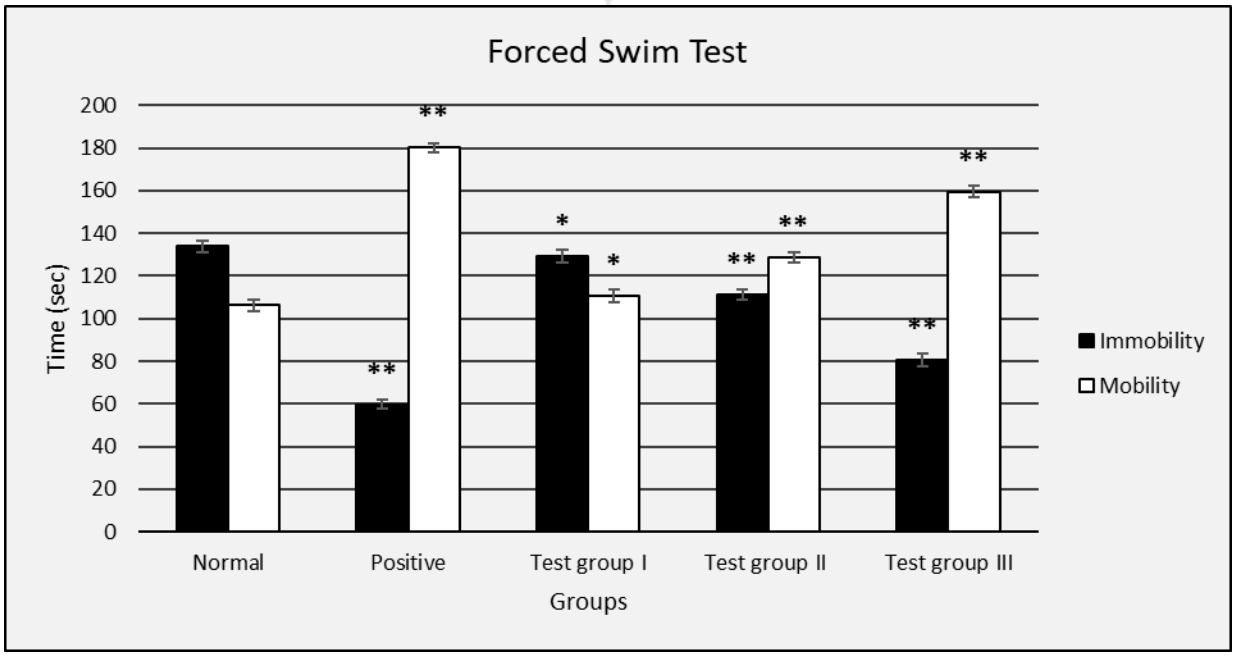

Figure No. 1: Effect of extract of leaves of *Carissa congesta* Wight on mobility and immobility time of FST in mice

TST

All the three test groups of the ethanolic extract of leaves of *Carissa congesta* Wight showed dose dependent decrease in immobility time when compared against normal control in tail suspension test (TST). Results of TST are shown in Table 5 and Fig. 2.

Table No. 5: Effect of extract of leaves of *Carissa congesta* Wight on mobility and immobility time of TST in mice

Treatment	Dose	Immobility time(sec)	Mobility time(sec)
Normal control (distilled water)	10 ml/kg (p.o.)	144 ± 2.09	96 ±2.09
Positive control (Diazepam)	2 mg/kg (i.p.)	60.33 ±2.16**	179.67 ±2.16**
Test 1 *Carissa congesta* Wight.	100 mg/kg (p.o.)	133.33 ±3.26**	106.67 ±3.26**
Test 2 *Carissa congesta* Wight.	200 mg/kg (p.o.)	100.33 ±2.16**	139.67 ±2.16**
Test 3 *Carissa congesta* Wight.	400 mg/kg (p.o.)	76.83 ±2.85**	163.17 ±2.85**

(Each value is given as Mean ± S.D. (n=6) one way ANOVA followed by Dunnett Multiple Comparisons Test; **p<0.01 when compared to the corresponding values of the normal control)

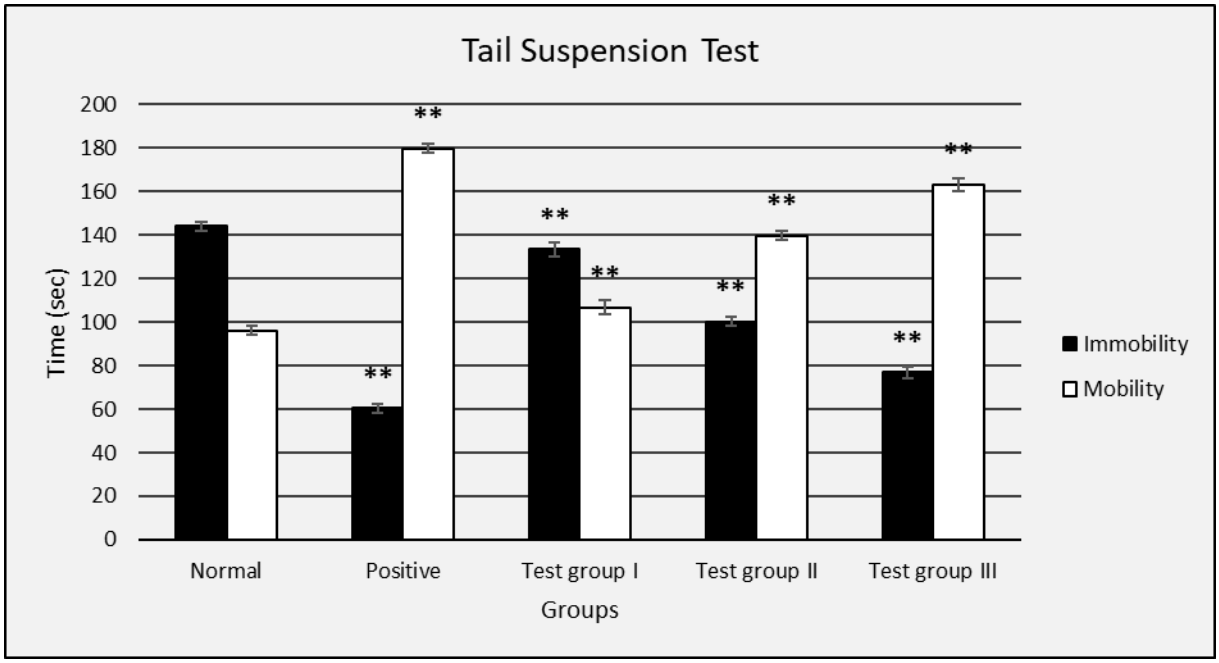

Figure No. 2: Effect of extract of leaves of *Carissa congesta* Wight on mobility and immobility time of TST in mice

DISCUSSION

Medicinal plant is widely used by the population of developing countries as alternative therapy because they are the potential sources of bioactive agents. In India, hundreds of plants are used traditionally for the management of various chronic diseases but unfortunately only a few of such medicinal plants have been made to evaluate the role of medicinal plants for their potential pharmacological activities in experimental mice and rats.

The treatment mainly focused in management of stress. Stress is associated to activate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of corticotropin releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN). CRH causes the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to secrete corticosterone which due to its lipophilic nature easily enters the brain. Within the brain the hormone acts at those sites where corticosteroid receptors are expressed such as in limbic areas. NA and corticosterone, stress exposure also leads to enhanced release of neuropeptides in the brain, such as vasopressin and corticotrophin releasing hormone (CRH). Phytochemical analysis of the leaves of *Carissa congesta* Wight

has shown the presence of triterpenoids and flavonoids. It has been seen that there is a direct relation between the triterpenoids, Flavonoids and their antistress activity.

At the initiation, in order to estimate the CNS profile of the test plant extract, they were screened in several preliminary behavioral animal models such as Forced swim test and tail suspension test. Significant reduction is an indication of its stress action while excessive reduction may indicate neurotoxicity too. Hence it is advisable to check the degree of the change in these parameters upon administration of drug.

The forced swim test (FST) or despair swim test and tail suspension test are most widely used tests for the evaluation of anti-stress property of novel compounds. In FST, mice were forced to swim in a restricted space from which there was no escape, and will, after periods of agitation, cease attempts to escape and become immobile. It is accepted that immobility seen in rodents during swimming reflects behavior despair as seen in human depression and that the antistress drugs are able to reduce the immobility time in mice.

In FST, animal forced to swim in water eventually assume a characteristic immobile posture which reflects a state of tiredness, fatigue reduced stamina or depressed mood. These signs represent the core symptoms observed in depressed patients and in individual under intense stress. Drugs with antistress property reduce the duration of immobility in animals. It has been well demonstrated that drugs with antistress activity increase swimming endurance. It was observed that there was a decrease in the immobility of the test doses compared to the normal control. Results of the FST indicate clearly that the leaves have the antistress properties. All three test doses showed dose dependent decrease in immobility time in FST and thus further supports its antistress potential.

The tail suspension test (TST) has been described by Cryan *et al.* (2005) as a facile means of evaluating potential antistress. The immobility displayed by rodents when subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair, which in turn may reflect depressive disorders in humans. Clinically effective Antistress reduces the immobility that mice display after active and unsuccessful attempts to escape when suspended by tail. It was observed that there was a decrease in the immobility of the test doses compared to the normal control. Results of the TST indicate clearly that the leaves have

the antistress properties. All three test doses showed dose dependent decrease in immobility time in TST and thus further supports its antistress potential.

There are reports to indicate that immobility, swimming and climbing behaviors are enhanced by different groups of antistress drugs. Nearly all antistress like NE-selective uptake inhibitors like desipramine (DMI) and maprotiline (MAP) enhances the climbing behaviors whereas the serotonin specific reuptake inhibitors (SSRIs) like fluoxetine (FLX), sertraline (SRT) and paroxetine (PRX) enhance swimming but not climbing behavior. However, both the types of antistress reduce immobility behavior.

It was observed from the results that the leaves of *Carissa congesta* Wight showed significant ($P<0.01$ and $P<0.05$) dose dependent decrease in the immobility time in FST and TST when compared against the normal control group. This implies that it contain active chemical constituents to elicit specific types of behavior in TST and FST through noradrenergic, serotonergic and dopaminergic systems and thereby acting as an antistress agent. It also suggests that the activation of these systems may depend on the concentration of the extract. Further, there was no remarkable change in the ambulatory behavior on chronic treatment of all the extracts at said dose levels. The ambulatory behavior decreased in comparison to the normal control group but no significant difference was found. This ensures that any increase in mobility observed in the TST, after treatments.

CONCLUSION

There is substantial evidence that triterpenoids and flavonoids play an active role in providing antistress activity. This study is an attempt to find out the alternative medication for treating chronic stress with single medication which was shown beneficial effect in animal models, may be useful for curing symptoms of stress. Hence rationally selected medicinal plant *Carissa congesta* Wight. The systematic pharmacological evaluation leads to conclude following,

- The Phytochemical tests of leaves revealed the presence of triterpenoids, flavonoids, steroids, glycosides, saponins and alkaloids.

- The initial behavioral observations did not revealed any gross change in the animal

behavior treated with the extracts of the plant.

It was observed from the results that *Carissa congesta* showed significant (P< 0.01) dose dependent decrease in the immobility time in FST and TST when compared with the normal control group in dose dependent manner. It was observed that the plant extracts possess antistress activity at higher dose levels.

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AUTHORS CONTRIBUTION

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Drx. (Mr.) Santosh Yadav collected the data, analyzed the data, all the laboratory work performed, wrote the introduction, discussion and the material and method part. Mr. Imtiyaz Ansari proof-read the whole manuscript as well as helps in designing and conducting the study.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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