

Ameliorating effect of *Gnetum gnemon* L. on hypothalamic pituitary adrenal axis during acute and chronic stress in rats

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There is an imperative pre-requisite to look for newer and safer drugs derived from natural resources, which will help in fighting stress. *Gnetum gnemon* L. (*Bago*), a versatile medicinal plant with a wide range of ethnobotanical utilizations, has been broadly used therapeutically and is becoming increasingly popular as nutraceutical agent. The present study was undertaken to evaluate the antistress or adaptogenic activity of *Gnetum gnemon*. Male Wistar rats were grouped as acute stress (AS) group, chronic stress (CS) group, vehicle control group and standard drug (Zeetress) treated group. The rats exposed to acute stress (AS) for 3 days and chronic stress (CS) for 7 days were treated with ethanolic extract of *Gnetum gnemon* (100 and 200 mg/kg p.o.) daily for (AS and CS), respectively. Water immersion stress was used in stress models. Ethanolic extract of *Gnetum gnemon* (100 and 200 mg/kg p.o.) reverted the elevated levels of ALT, AST, plasma glucose, cholesterol and creatine kinase and triglyceride level. In the extract treated groups, the size of the spleen was normalized than the stress control group. The size of the hypertrophied adrenal glands was reduced due to its putative adaptogenic property.

Keywords: Adaptogen, *Gnetum gnemon*, HPA axis, Stilbenoids, Zeetress

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Stress involves in the etiopathogenesis of a diverse range of diseases, extending from psychiatric disorders such as depression and anxiety, immunosuppression, endocrine disorders including diabetes mellitus, male sexual dysfunction, cognitive dysfunctions, peptic ulcer, hypertension and ulcerative colitis¹. Drugs like benzodiazepines, certain CNS stimulants such as amphetamines and caffeine as well as some anabolic steroids are routinely used by people to combat stress. However, the incidence of toxicity and dependence has limited the therapeutic usefulness of these drugs. The concept of treating stress-related conditions with medicinal plants is familiar to most traditional healing models throughout the world. Therefore, there is an imperative prerequisite to look for newer and safer drugs derived from natural resources, which will help in fighting stress. Several plants have been reported which have

adaptogenic and rejuvenating properties and are being investigated for remedies for a number of disorders including antistress (adaptogenic) activity².

Gnetum gnemon (*Bago*), a versatile medicinal plant with a wide range of ethnobotanical utilizations, has been broadly used therapeutically and is becoming increasingly popular. It is a genus of *Gnetum*, indigenous to South east Asia, the Western Pacific Ocean islands, Indonesia and Malaysia to the Philippines and Fiji³⁻⁵. *Melinjo* is the acquainted Indonesian name for this tiny tree and has been consumed as a safety foodstuff for centuries. The fruits and seeds of *Gnetum gnemon* are consumed for their nutrient values in addition to its use in traditional medicines⁶. *Gnetum gnemon* is a safe food additive and can be utilized effectively as a basic raw material to develop a novel nutritious functional food. Nutritional analysis showed that the seed flour abundant in protein (19.0 g/100 g), crude fibre (8.66 g/100 g), carbohydrates (64.1 %), total dietary

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fibre (14.5 %) and encompassed adequate amounts of essential amino acids, fatty acids and minerals. Total phenols (15.1 and 12.6 mg GAE/100 g), tannins (35.6 and 16.1 mg CE/100 g) and flavonoids (709 and 81.6 mg CEQ/100 g) were also estimated⁶. The most profuse phytoconstituents has been reported to be saponins, tannins, flavonoids and stilbenoids⁷⁻⁸. Scientific data revealed that *Gnetum gnemon* has antimicrobial, antitoxic, antioxidant, anti-inflammation sensing and antisenescence properties owing to these phytoconstituents⁹⁻¹². Stilbenoids were found to be responsible for the pharmacological effects of *Gnetum gnemon* like inhibitory effect on tyrosinase activity, melanin biosynthesis and on multiple angiogenesis¹³. Furthermore, clinical studies proposed that stilbenoids were beneficial in managing diabetes and cardiovascular diseases. The plant is extremely popular among few tribes of North east India, mainly in Nagaland, Manipur and Karbi Anlong districts, where the leaves of this plant is their regular part of diet be it green vegetable, or soup or curry or as spice. Seeds are used for making *pakor*as in Indonesia.

Detailed pharmacological studies can fill up the gap for development of a novel compound with curative as well as nutritive properties. Hence, it was thought prudent to experimentally appraise the potential usefulness of leaves of *Gnetum gnemon* for its antistress and adaptogenic activity as well.

Methodology

Drugs and chemicals

Zeetress, the standard adaptogenic drug was obtained from Sigma-Aldrich (St. Louis, MO, USA). All compounds were dissolved in distilled water and Tween 80 (0.1 %) solution. Drugs were prepared fresh daily before administration.

Collection of plant materials and preparation of extract

In North east India, *Gnetum gnemon* is popularly known by *Karbi* name *Hanthu* or Assamese name *Letera*¹⁴. The seeds are also known as *Belinjau* in some parts of the world. The fresh leaves of the plants were collected from the local market of Silanijan, Karbi Anglong district, Assam. It was identified by Taxonomist Dr. Iswar Chandra Barua, Assam Agricultural University, Jorhat, Assam and a voucher specimen (Barua 4704 dated 3.7.13) was deposited. Herbaria of the plant was prepared and authenticated from Central National Herbarium, BSI, Howrah, West Bengal. Voucher specimen (Bar code no 00000 CAL 27000) was deposited at AAU, Khanapara (Fig. 1).

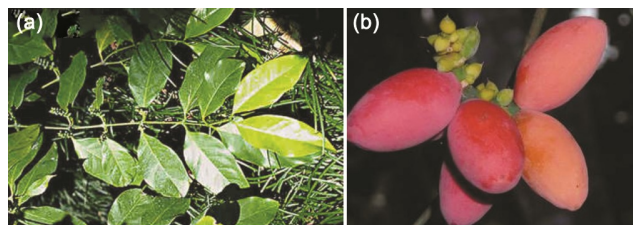


Fig. 1 — Leaves and fruits/seeds from *Gnetum gnemon*

Leaves of *Gnetum gnemon* were cleaned from extraneous materials, washed, shade dried, powdered mechanically, weighed, and stored in airtight container. About 250 g of powdered material was soaked in 1000 mL ethanol for 72 h in a beaker and mixture was stirred every 18 h using a sterile glass rod. Filtrate was obtained three times with the help of Whatman filter paper no. 1 and the solvent was removed by rotary evaporator (Buchi R-210, BUCHI Labortechnik AG, Meierseggstrasse Switzerland) under reduced pressure at < 45 °C temperature leaving a dark brown residue. It was stored in airtight container at 4 °C until use.

Animals

Adult male Wistar rats weighing between 170 to 200 g were used for the experiment. The animals were allowed for acclimatization to the condition at least for 7 days. Rats were housed three to four per cage at a constant temperature (22 ± 2 °C) and 12/12 hr light/dark (8:00 am to 8:00 pm) cycle and were fed standard laboratory food, water was given *ad libitum*. All experiments were performed according to current guidelines for the care of laboratory animals by IAEC (No.773/ac/CPCSEA/FVSc, AAU/IAEC/10-11/72).

Phytochemical screening

Ethanol extract of *Gnetum gnemon* was subjected to preliminary, qualitative and phytochemical investigations for the presence of various active principles as per standard method¹⁵.

Experimental design

The rats were divided into control group, acute stress (AS) and chronic stress (CS) groups and extract treated groups both for AS and CS, standard drug (Zeetress) treated group, consisting of 6 animals in each group. The AS groups were fed with ethanolic extracts of *Gnetum gnemon* (100 and 200 mg/kg p.o.) daily for 3 days. Control group of rats fed with vehicle for the same number of treatment days but were not subjected to stress to obtain standard data for different parameters. On the second day, after feeding drug or vehicle, animals were fasted overnight with

free access to water. On the third day, 45 min after feeding the drug or vehicle, rats were allowed to swim for 20 min except the control group. In CS, the drugs were fed daily 45 min prior to swimming, up to seven consecutive days except that the rats were fasted overnight on the sixth day after completion of the experimental regimens of drug feeding and swimming.

Among the numerous methods employed, water immersion stress was used as it is considered to be one of the best model of stress which provides both emotional stress (despair behavior) as well as physiological (vigorous muscular activity) stress to the rodents and has been used extensively and accepted widely¹⁶. The rats were anaesthetized with xylazine (13 mg/kg i.p.)¹⁷, the commonly used anaesthetic, after stress; blood was collected in BD Vacutainer K2 EDTA 3.6 mg REF 367841 through cardiac puncture. The collected blood was centrifuged at 2000 rpm for 20 min at 4 °C. Plasma was used to estimate glucose, triglycerides, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK) using spectrophotometer (Multiskan GO, Thermo scientific) with their respective kits (Siemen Ltd.). The adrenals, spleen and thymus were dissected under anaesthesia and weighed after removal of adhering tissues.

Sub acute toxicity study

The study was directed as per the protocol of Organization for Economic Cooperation and Development (OECD, 2007) guideline for testing of chemicals, Acute toxic class method (OECD 423). Previous reports using 5000 mg/kg oral dose showed no mortality with this plant extract¹⁸. Limit test was done using 2000 mg/kg oral dose in rats and observed for any gross abnormality, mortality following next 24 h. The animals (male Wistar rats) were fasted overnight and ethanolic extract of *Gnetum gnemon* was administered orally at the dose levels 250, 500, 1000, 1500 and 2000 mg/kg body weight p.o. respectively in five different groups along with a vehicle control group. Animals were observed individually after dosing for a total of 4 weeks for any clinical signs of toxicity and mortality. Any change in body weight, food and water intake was recorded. Food and water consumption was measured every day till the end of study period. Food containers were filled with 50 g of the pelleted mice chow, and food intake was quantified daily. Consumption of food were recorded by subtracting the food remaining in

the food container and on the cage floor from the amount of food measured at the preceding time point. Food spillage in the cage was ignored because it has been previously reported to be similar among rats/mice and generally weigh less than 1 % of the food consumed¹⁹.

The body weight changes were recorded weekly while food and water intake were observed daily.

Statistical analysis

Results are presented as Mean \pm SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) among different treatment groups followed by Tukey–Kramer test (Graph Pad Prism 5.0). $p < 0.05$ was considered to indicate statistical significance.

Results

Phytochemical screening

The phytochemical screening of ethanolic extract of *Gnetum gnemon* showed the presence of diterpenes and triterpenes by Salkowski's test and Liberman Buchardt's test; glycosides by Anthraquinone test and phenolics by ferric chloride test. The quantitative tests for phenolics by Folin Ciocalteu's test and flavonoids²⁰ were carried out and the yield were found to be 0.148 ± 0.025 mg tannic acid/g of dry plant material and 0.011 ± 0.006 mg/g quercetin equivalent, respectively.

Sub acute toxicity study

The animals did not show any sign of gross abnormality or mortality in 24 h limit test with 2000 mg/kg oral dose. Based upon this, two doses, viz. 100 and 200 mg/kg oral doses were selected for the study. At all dose levels of ethanolic extract of *Gnetum gnemon*, the test animals showed no significant variations in behavior.

Influence of treatment on AS- and CS-induced modifications in organ weight

Exposure to AS and CS significantly ($p < 0.05$) augmented the weight of adrenal gland (0.0238 ± 0.00011 g and 0.0295 ± 0.00016 g) as compared to their respective non stress control groups (0.0113 ± 0.00012 g). Pre-treatment with ethanolic extract of *Gnetum gnemon* at 100 and 200 mg/kg po (0.0237 ± 0.00021 g and 0.0151 ± 0.0001 g) significantly ($p < 0.05$) reduced the weight of adrenal gland weight in CS induced group which was not very effective in restoration of size of adrenal gland in AS induced group (Tables 1&2).

Table 1 — Data showing effects of various extracts on the weights of adrenal gland and spleen along with various biochemical parameters in acute stress in rats. (N = 6). Results are represented as mean ± S.E.M. with n = 6 in each group. * p < 0.05 as compared to the control group (for AS), # p < 0.05 as compared to the AS, ** p < 0.05 as compared to the control group (for AS), ## p < 0.05 as compared to the AS group.

Parameters	Vehicle control	Acute Stress	<i>G. gnemon</i> 100 mg /kg p.o.	<i>G. gnemon</i> 200 mg /kg p.o.	Standard (zeetress)
Adrenal gland (g)	0.0113 ± 0.00012 [#]	0.0238±0.004*	0.0237 ± 0.00021 ^{**}	0.0151± 0.0001 ^{**}	0.0131±0.001 ^{**##}
Spleen (g)	0.570 ± 0.0012 [#]	0.292 ± 0.0016*	0.393±0.34 ^{**}	0.399 ± 0.003 ^{**}	0.523±.009 ^{**##}
ALT activity (IU/L)	48.506 ± 1.745 [#]	72.848 ± 3.434*	73.66±1.97 [#]	42.590 ± 1.406 [#]	47.00±4.872 [#]
AST activity (IU/L)	143.646 ± 2.443 [#]	226.635 ± 6.853*	177.50±5.60 [#]	145.480 ± 2.130 [#]	142.50±3.25 [#]
Glucose (mg/dL)	64.16±4.028	139.348 ± 2.779*	61.16±14.09 [#]	66.033 ± 2.680 [#]	68.000±6.303 [#]
Cholesterol (mg/dL)	33.590 ± 0.792 [#]	66.908 ± 2.753*	62.500±8.330*	47.833±2.750 [#]	24.417±3.946 [#]
Triglycerides (mg/dL)	85.280 ± 3.106 [#]	40.068 ± 2.687*	77.391 ± 4.666 ^{**}	64.166 ± 4.808 ^{**}	24.417±3.946 ^{**##}
Creatine kinase (mg/dL)	235.16±23.155 [#]	1602.222± 30.910*	240.848 ± 5.672 [#]	254.023 ± 8.538 [#]	378.89±29.891 ^{**##}

Table 2 — Data showing effects of various extracts on the weights of adrenal gland, spleen along with various biochemical parameters in chronic stress in rats. (N = 6). Results are represented as mean ± S.E.M. with n = 6 in each group. * p < 0.05 as compared to the control group (for CS), # p < 0.05 as compared to the CS, ** p < 0.05 as compared to the control group (for CS), ## p < 0.05 as compared to the CS group.

Parameters	Vehicle control	Chronic stress	<i>G. gnemon</i> 100 mg /kg p.o.	<i>G. gnemon</i> 200 mg /kg p.o.	Standard (zeetress)
Adrenal gland (g)	0.0113 ± 0.00012 ^{##}	0.0295± 0.00016 ^{**}	0.0290±.002 ^{**##}	0.0230±0.003 ^{**##}	0.0131±0.001 ^{**##}
Spleen (g)	0.570 ± 0.0012 ^{##}	0.292 ± 0.0016 ^{**}	0.393±0.34 ^{**##}	0.434 ± 0.0027 ^{**##}	0.523±0.009 ^{**##}
ALT activity (IU/L)	48.506 ± 1.745 ^{##}	89.741 ± 2.472 ^{**}	73.66±1.97 ^{**##}	52.405 ± 0.708 ^{##}	47.00±4.872 ^{**##}
AST activity (IU/L)	143.646 ± 2.443 ^{##}	322.863 ± 4.348 ^{**}	177.50±5.60 ^{##}	140.00±1.36 ^{##}	142.50±3.25 ^{##}
Glucose (mg/dL)	64.16±4.028 ^{##}	136.35±2.081 ^{**}	61.16±14.09 ^{**##}	87.390 ± 1.643 ^{**##}	68.000±6.303 ^{**##}
Cholesterol (mg/dL)	33.590 ± 0.792 ^{##}	105.041 ± 3.801 ^{**}	62.500±8.330 ^{**##}	47.833±2.750 ^{**##}	24.417±3.946 ^{**##}
Creatine kinase (mg/dL)	235.16±23.155	1784 ± 27.135 ^{**}	296.835 ± 12.815 ^{##}	255.128 ± 13.473 ^{##}	378.89±29.891 ^{**##}

After exposure to AS and CS, a considerable reduction (p < 0.05) in the weight of spleen (0.292 ± 0.0016 g) of the animals was noticed as compared to the respective control groups (0.570 ± 0.0012 g). Pretreatment with *Gnetum gnemon* extract at 100 and 200 mg/kg significantly (p < 0.05) increased the spleen weight and the effect was better in CS induced group (0.434 ± 0.0027 g) than that of AS group (0.399 ± 0.003 g). No significant change in the weight of thymus was observed in AS and CS treated animals. The results are shown in Tables 1&2.

Influence of treatment on AS and CS induced alterations in biochemical parameters

A considerable increase (p < 0.05) in the plasma ALT level was observed in the AS (72.848 ± 3.434 IU/L) and CS (89.741 ± 2.472 IU/L) induced group as compared to the non stress groups (48.506 ± 1.745 IU/L). *Gnetum gnemon* at 200 mg/kg p.o. significantly (p < 0.05) decreased the ALT level in both AS (42.590 ± 1.406 IU/L) and CS (52.405 ± 0.708 IU/L) induced groups. The effect was better than that of *Gnetum gnemon* (100 mg/kg p.o.) and the standard drug treated group, which is shown in Tables 1&2.

Exposure to AS and CS to the rats resulted in a significant (p < 0.05) escalation in plasma AST level (226.635 ± 6.853 IU/L and 322.863 ± 4.348 IU/L) as compared to the corresponding control groups (143.646 ± 2.443 IU/L). Pretreatment with *Gnetum gnemon* at 100 and 200 mg/kg p.o., significantly (p < 0.05) decreased the AST level (145.480 ± 2.130 IU/L) as compared to AS induced group (226.635 ± 6.853 IU/L). Furthermore, pretreatment with *Gnetum gnemon* at 100 and 200 mg/kg p.o. significantly (p < 0.05) decreased the AST level in AS and CS induced groups. The effect was better in AS induced as compared to CS induced group.

Acute and chronic exposure to stress resulted in significant (p < 0.05) increase in the plasma level of cholesterol (66.908 ± 2.753 mg/dL and 105.041 ± 3.801 mg/dL) as compared to the respective non stress groups (33.590 ± 0.792 mg/dL). Pretreatment with *Gnetum gnemon* with 100 and 200 mg/kg p.o. dose dependently resulted in significant (p < 0.05) decrease in the plasma cholesterol level both in AS and CS induced groups (Tables 1&2).

Likewise, exposure to AS significantly ($p < 0.05$) decreased the plasma level of triglyceride (40.068 ± 2.687 mg/dL) as compared to respective non stress control (85.280 ± 3.106 mg/dL). Pre-treatment with *Gnetum gnemon* at 100 and 200 mg/kg p.o. showed significant ($p < 0.05$) increase in the plasma triglyceride level (77.391 ± 4.666 mg/dL and 64.166 ± 4.808 mg/dL) after AS exposure (Table 1).

Obviously, significant ($p < 0.05$) increase in the plasma level of glucose was detected in AS (139.348 ± 2.779 mg/dL) and CS (165.206 ± 1.789 mg/dL) induced groups, whereas, pretreatment with *Gnetum gnemon* at 100 and 200 mg/kg p.o. significantly ($p < 0.05$) reverted the elevated glucose level towards normal after AS (66.033 ± 2.680 mg/dL) and CS (87.390 ± 1.643 mg/dL) exposures. This is represented in Tables 1&2, respectively.

Likewise, a significant ($p < 0.05$) increase in the plasma level of CK activity as compared to non stress control groups was observed in AS (1602.222 ± 30.910 mg/dL) and CS (1784 ± 27.135 mg/dL) induced control group of animals. Pretreatment with *Gnetum gnemon* with 100 and 200 mg/kg p.o. showed a significant ($p < 0.05$) trend of decrease in the CK activity in AS (240.848 ± 5.672 mg/dL and 254.023 ± 8.538 mg/dL) and CS (296.835 ± 12.815 mg/dL and 255.128 ± 13.473 mg/dL) induced groups (Tables 1&2).

Discussion

A multifaceted relationship exists amongst stressful circumstances, mind and body's response to stress, and the inception of clinical despair. Chronic stress is a condition which is mostly characterized by motor impairment, cognitive dysfunction and anxiety like behavior, which is not significantly improved by rest²⁰. However, the aetiology of chronic stress still remains uncertain. Alterations of the hypothalamic–pituitary–adrenal (HPA) axis ought to be revealed in patients with chronic fatigue stress²¹. It is important to note that *Gnetum gnemon* has not yet been reported as an adaptogen. Therefore, interesting avenues need to be opened up to exploit the therapeutic effects of the plant in the treatment of different disorders. Reports demonstrated that the seed extract of *Gnetum gnemon* devours no health risk in acute and sub chronic oral toxicity study and the LD₅₀ exceeded 5000 mg/kg bw¹⁸. In our sub acute toxicity study, there was no mortality up to a maximum dose of 2000 mg/kg body weight of ethanolic extract of *Gnetum gnemon* after oral administration. Since no remarkable changes

were observed in animal behavior, body weight and organ weight at all dose levels in treated rats as compared to control group, it can be deduced that *Gnetum gnemon* is non toxic at the doses administered.

The penalty area of the present study was to investigate the impact of altered organ weights and pharmacologically increased AST, ALT, CK and glucose level on the course of the current mood state of animals in acute and chronic stress paradigm. We anticipated that *Gnetum gnemon* pretreatment might have a stress-buffering effect on mood. For the period of stress, the nerve terminals quicken the recruitment of lymphocytes to blood from spleen, which is a main storage pool of lymphocytes²². This results in the squeezing of the spleen causing reduction in weight observed in both AS and CS exposures. Our study also perceived subsequent increase in the stress induced reduction of weight of spleen in rats.

Stress-induced adrenal hypertrophy was found both in AS and CS. However, the effect was seen more after CS exposure. This may be the result of activation of the HPA axis, which is known to be highly responsive to stress and is regarded as one of the primary mechanism by which an organism activates its defence system against stressful trials²³⁻²⁴. In our study, the prolonged activation of HPA axis resulted in an increase in the adrenal hypertrophy in CS as compared to AS. The increased requirement of adrenal cortical hormones during stress may be one of the reasons for increased adrenal weights in stress control group. *Gnetum gnemon* pretreatment in both doses caused reversion of escalated weight of adrenal gland and hypertrophy of spleen caused due to the stress thus hindering the basic signs of stress reaction. Corticosterone from adrenal cortex and epinephrine from adrenal medulla are considered to be necessary manipulators in the body against stress response and help in combating stress²⁵⁻²⁶.

In the present study, there was significant increase in ALT, AST, CK and blood glucose in both AS and CS exposure which can be the consequence of AS and CS induced oozing of corticosterone from cortex, epinephrine from medulla, sympathetic nerve terminals providing substrate for energy metabolism and the assurance of availability of ATP demand in the CNS, muscles and other organs of demand. The acute demand of glucose was fulfilled by the increase in glucogenolysis from liver throughout AS. However, this source exhausts during CS. Consequently, it utilizes fat as a secondary substrate

and activating gluconeogenesis in response to corticosterone. ALT and AST enzymes catalyze the transfer of the γ -amino groups of alanine and aspartate, respectively, to the α -keto group of ketoglutarate, leading to the formation of oxaloacetic acid and pyruvic acid. As a result of transamination, amino acid can enter the citric acid cycle and then function in the intermediary metabolism of carbohydrate and lipids²⁷. There was significant decrease in the plasma triglyceride level after AS, which may be due to enhanced activity of lipoprotein lipase²⁸⁻²⁹ or decrease in endogenous cholesterol as well as triglyceride production³⁰ and *Gnetum gnemon* (100 mg/kg) was found to be more effective in reverting the alteration of these markers back to normal following AS. But no significant effect was observed in the level of plasma triglycerides after CS exposures. *Panax quinquefolium*, *Withania somnifera*³¹ and *Ocimum sanctum*³² has been reported to be effective against stress-induced gastric ulcers and adrenal gland hypertrophy, and *Gnetum gnemon* also showed similar effect in our study.

Conclusion

In conclusion, the present investigation indicates that ethanolic extract of *Gnetum gnemon* (100 and 200 mg/kg bw) has shown significant adaptogenic activity by its extenuating effects on several acute and chronic stress-induced biochemical and physiological parameters comparable to that of Zeetress, the conventional anti stress agent. The phytoconstituents (mainly phenolics and flavonoids) present in the active extracts play an important role in these pharmacological activities. Since the plant is popular among the local natives owing to its high nutritive values, this novel plant extract can be a very potential and putative agent for exploiting as a new anti stress compound.

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