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Research Article

Screening of Synergistic Antistress Activity of *Phyllanthus Niruri* Leaves and *Sapindus mukorossi* Fruits

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ABSTRACT

Phyllanthus niruri leaves and *Sapindus mukorossi* fruits have been traditionally used as anti-inflammatory, anti-pyretic and analgesic, anti-fungal, anti-microbial, anti-bacterial and antiparasitic, expectorant, emetic, anti-cancer, mood elevator, and as hepatoprotective. The present study was designed to investigate the anti-stress activity of combined extract of *P. niruri* leaves and *S. mukorossi* fruits employing various activities using experimental animals. Rats and Mice were separated into five groups, consisting of six animals each. Methanolic extract of *P. niruri* leaves (MEPNL), aqueous extract of *S. mukorossi* fruit (AESMF), and combined extract (MEPNL+AESMF) were prepared and anti-stress activity was investigated by Anoxia induced stress in mice, Immobilization stress in rats, and Swimming endurance test in mice. Combined extract (400 mg/kg, p.o.) exhibited remarkable effect as it delayed onset of convulsion time in anoxia stress model when equated to individual extracts. In swimming endurance test, combined extract (400 mg/kg, p.o.) displayed significant effect as it enhanced swimming time in mice when compared to MEPNL (400 mg/kg, p.o) and AESMF (400 mg/kg, p.o). Combined extract has remarkably reduced the serum levels of glucose, triglycerides, cholesterol, blood urea nitrogen, and cortisol in rats as equated to control. Combination of extracts has produced remarkable and protective effects on organs such as liver, adrenal gland, spleen, and testes. Present study showed that the combined extract of *P. niruri* leaves and *S. mukorossi* fruits possessed significant anti-stress action, which was found to be higher than the individual extracts. This indicates the synergistic anti-stress efficacy of the combination of extracts. However, future studies are essential to focus on the cellular mechanism of quantitative phytochemical constituents and ascertain the precise mode of action involved in its anti-stress property.

Introduction

Stress is the body's response to a particular demand. It can occur as a result of events that cause frustration, nervousnes or anger. Stress is a condition that stimulates a characteristic biological effect. Stress is a primary factor in the breakdown of homeostatic functional balance, in that biological systems are in a persistent rate of flux, continuously compensating to the responses of both external and internal stimuli.[1] Stress is the nonspecific response of the body to any demand made upon it. Stress is the mental, physical, and chemical reaction of the body that occurs when we get confused, excited, or feel unsafe.[2] Stress has been postulated that is involved in the pathogenesis of various diseases ranging from psychiatric diseases such as anxiety, immunosuppression, depression, and endocrine diseases such as diabetes mellitus, cognitive dysfunctions, male sexual dysfunctions, peptic ulcer, hypertension, and ulcerative colitis.[3] In this modern era, stress has become an integral part of human life. It is vital that stress is kept under control and normal functioning is not hampered due to excessive stress. Stress is considered to be any condition that results in perturbation of body's homeostasis. If the level of stress is extreme, the homeostatic mechanism of the organism becomes deficit

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and the survival of the organism is threatened.[4] The leaves of *P. niruri* plant are reported to have multiple therapeutic properties such as anti-inflammatory, antipyretic and analgesic, anti-fungal, anti-microbial, anti-bacterial and antiparasitic, anti-cancer, and hepatoprotective activity.[5] The fruit of the *S. mukorossi* plant is reported to have expectorant, emetic, hepatoprotective, mood-elevating, and abortifacient effects.[6] Adaptogens are the plants or biologically active substances that induce a state of nonspecific increase of resistance of the organism to aversive stimuli, which threaten the internal homeostasis and improve the physical endurance for doing work in difficult environmental conditions. Adaptogens have been claimed to arrest the aging process and deterioration in physical and mental performance.[7] Research study on herbal drug shows less or no side effect than the allopathic drug. However, no existing pharmacological data indicate the usefulness of a combination of *P. niruri* leaves and *S. mukorossi* fruits extract against stress. Therefore, this study was carried out to evaluate the anti-stress activity of combined extract of *P. niruri* leaves and *S. mukorossi* fruits and comparison of anti-stress efficacy of combined extract with individual extracts. Further, there is a scope to develop as nutraceutical tablets in the effective treatment of anti-stress.

Materials and Methods

Collection and Authentification of Plant Material

For the present study, the *P. niruri* leaves and *S. mukorossi* fruits were collected from the vicinity of the Meerpet, Saroornagar, Hyderabad. Sample specimens of *P. niruri* leaves and *S. mukorossi* fruits were deposited in a polythene bag. The sample specimens were kept in fresh condition by adding 2% formalin. Plant materials were identified and authenticated by Dr. K. Venkata Ratnam, Rayalaseema University, Kurnool, Andhra Pradesh.

Extraction

The *P. niruri* leaves and *S. mukorossi* fruits were shade dried separately and mechanically reduced to a coarse powder. The weight of the coarse powders of *Phyllanthus* leaves and *Sapindus* fruits were found to be 1450 g and 1368 g. The powders were subjected to hot continuous successive extraction in a Soxhlet apparatus with solvents in the increasing order of polarity using petroleum ether, ethyl acetate, acetone, methanol, and water under controlled temperature (50–60°C). Thus, the extracts were concentrated in a vacuum rotary evaporator, and extracts were kept in desiccators for further use.

Phytochemical Screening

Phytochemical qualitative analysis was performed by subjecting the crude extracts for identification tests to detect the presence of flavonoids, glycosides, alkaloids, carbohydrates, fixed oils, tannoids, phytosterols, proteins, amino acids, lignins, phenolic compounds, saponins, gums and mucilages.[8]

The MEPNL and AESMF were found to possess a significant number of active constituents are selected for anti-oxidant activity. The combined extract was prepared by mixing the extracts of *P. niruri* leaves and *S. mukorossi* fruits in an equal ratio (1:1).[9]

Fig. 1: Anoxia stress tolerance inmice

Fig. 2: Immobilisation stress in rats

Table 1: Experimental animals used in different groups in various stress models

Groups	*Anoxia stress tolerance model*	*Immobilization stress model*	*Swimming endurance model*
Positive control	6 mice	6 rats	6 mice
Standard	6 mice	6 rats	6 mice
Test group I	6 mice	6 rats	6 mice
Test group II	6 mice	6 rats	6 mice

Animals

Wistar rats (180–200 g) and Mice (20–25 g) were procured from Sainath Agencies, Musheerabad, Hyderabad, A.P., India (282/99/CPCSEA) and housed in the animal facility of the institution. After randomly dividing the animals into different groups, the rats were accustomed for one month before the experiment. Animals were caged in polypropylene cages and preserved under standard environmental conditions such as temperature (26 ± 2°C), relative humidity (45–55%) and 12 hours dark/light cycle. The animals were fed with rat pellet diet (Golden Mohur Lipton India Ltd.) and water *ad libitum.* The study protocol was approved by the institutional animal ethical committee.

Single-dose Oral Acute Toxicity for one Week with Gross Behavioral Study

The Acute toxicity evaluation of MEPNL and AESMF was performed based on OECD guidelines 423 by using mice, and fixed-dose studies were selected where the limit dose is 2000 mg/kg.

Experimental Methods

Anoxia Stress Tolerance Test

Mice were randomly separated into five groups, each group consisting of six mice. Positive control was treated with 0.9% Sodium chloride (10 mL/kg, p.o.), while the standard group received extracts of *Ashwagandha* (100 mg/kg, p.o.). Treatment groups were served with MEPNL, (400 mg/kg, p.o), AESMF, (400 mg/kg, p.o) and combined extract (MEPNL + AESMF, 400 mg/kg, p.o) for 21 days. On the 7th, 14th, and 21st days mice were subjected to anoxia stress by placing independently in a hermetic vessel of 1-liter sufficiency. The time period from the animal entry into the vessel and the first convulsion presentation is considered time of anoxia tolerance.[10-11]

Group 1: Positive Control (Normal saline 10 ml/kg, p.o.)
Group 2: Standard (*Withania somnifera* extract, 100 mg/kg, p.o.)
Group 3: MEPNL (Methanolic extract of *P. niruri* leaves, 400 mg/kg, p.o.)
Group 4: AESMF (Aqueous extract of *S. mukorossi* fruits, 400 mg/kg, p.o.)
Group 5: Combined extract (MEPNL+ AESMF, 400 mg/kg, p.o)

Immobilisation Stress

Rats were randomly separated into five groups, each group composed of six rats. The treatment was induced for 10 days, 1-hour prior to the stress exposure. Stress was generated by immobilizing rats with head downwards, supine position by arranging the forelimbs and hind limbs to a wooden board inclined at 600° angles, daily for 2 hours (Time schedule-11 A.M to 1 P.M) for a span of 10 days. The positive control group was treated with normal saline (10 mL/kg, p.o.) while the standard group was treated with extracts of *W. somnifera* (100 mg/kg, p.o.) for 10 days. Treatment group includes MEPNL (400 mg/kg, p.o), AESMF (400 mg/kg, p.o.) and combined extract (MEPNL + AESMF, 400 mg/kg, p.o) for 10 days.[12]

At the end of 10th-day animals (4 groups of 6 animals each) were terminated at the end of experimentation and blood was accumulated by cardiac puncture under the influence of ether anesthesia (mild) employing disposable syringe and needle for evaluation of serum biochemical parameters such as, glucose, triglycerides, cholesterol, blood urea nitrogen (BUN) and cortisol.[13]

The organs such as liver, adrenal gland, spleen and testes were cleansed with alcohol and their weights were noted per 100 g body weight of rat (4 groups of 6 animals each).

Swimming Endurance Test

Mice were randomly separated into five groups, each group is composed of six animals. Positive control was treated with 0.9% Sodium chloride (Normal saline, 10 mL/kg, p.o.) while standard group received extracts of *W. somnifera* (100 mg/kg, p.o.) for 7 days. Treatment groups were treated with MEPNL (400 mg/kg, p.o), AESMF (400 mg/kg, p.o) and combined extract (MEPNL + AESMF, 400 mg/kg, p.o) for one week. Swimming test was performed on 7th day 1 hr after oral administration of drug, employing polypropylene vessel (45 × 40 × 30 cm) with 20 cm water level. The end point was taken when animal remained at the bottom of the tank due to drowning. The mean swimming time for each group was calculated.[14]

Statistical Analysis

All values were asserted as Mean± SEM for all the experimental models.

Data was interpreted by following assay:

i. ANOVA (one way)continued by Dunnett's test.
ii. The results were regarded to be statistically significant when $p < 0.05$

Fig. 3: Swimming endurance test in mice

RESULTS

Phytochemical Investigations

Preliminary phytochemical investigation of MEPNL and AESMF indicates the presence of flavonoids, Saponins, alkaloids, tannins, carbohydrates, glycosides, fixed oils, phenolics, fats, and lignins. MEPNL and AESMF were selected for *in vivo* anti-stress activity. Flavonoids are detected more in MEPNL, and flavonoids are reported to have mood-elevating properties. Saponins are detected *S. mukorossi* with greater clarity in aqueous extract of fruit, and saponins are reported to be responsible for the anti-oxidant and anti-stress activity.

Preparation of combined extract of *P. niruri* leaves and *S. mukorossi* fruit

Both the leaf and fruit extracts were combined in equal ratios (1:1) i.e 5 grams of *P. niruri* leaf extract with 5 grams of *S. mukorossi* fruit extract.

Acute toxicity studies of MEPNL and AESMF

The MEPNL and AESMF were administered to mice at doses 5, 50, 300, and 2000 mg/kg with an oral syringe did not display any symptoms of toxicity. The rats were examined for two weeks twice a day has not exhibited toxic signs. Hence oral LD_{50} of MEPNL and AESMF were finalized to surpass 2000 mg/kg. Therefore 2000 mg/kg was regarded as the safest higher dose for MEPNL and AESMF and $1/5^{th}$ of 2000 mg/kg, i.e., 400 mg/kg (higher dose) of MEPNL, AESMF, and combined extract (MEPNL+AESMF) were preferred for the further anti-stress activity.

Effects of Methanolic Extract of *P. niruri* leaves (MEPNL), Aqueous Extract of *S. mukorossi* fruit (AESMF), and Combined Extract (MEPNL+AESMF) on Anoxia Stress Tolerance Test in Albino Mice

As shown in result Table 2 and Fig. 4, combined extract (MEPNL+AESMF) at dose 400 mg/kg p.o. showed a significant and dose-dependent increase in the convulsion time (189.17 ± 1.54 minutes), whereas MEPNL and AESMF (400 mg/kg orally) showed mild effect (MEPNL 162.66 ± 0.67 minutes, AESMF 156.16 ± 1.73 minutes) when equated with control. The combined extract has exhibited a greater increase in convulsion time in mice when compared to individual extracts. Similarly, *W. somnifera* (100 mg/kg orally) treated group exhibited a statistically remarkable effect by an increase in convulsion time (189.17 ± 1.54 minutes) as compared to the control.

Effects of Methanolic Extract of *P. niruri* leaves (MEPNL), Aqueous Extract of *S. mukorossi* Fruit (AESMF) and Combined Extract (MEPNL+AESMF) on Biochemical Parameters in Immobilisation Stress-induced Albino Rats

As shown in result Table 3 and Figs. 5 and 6, the combined extract (MEPNL + AESMF, 400 mg/kg, p.o) showed a significant reduction in the glucose level, cholesterol level, triglycerides level, blood urea nitrogen level, and cortisol level when compared with control. The reduction of biochemical parameters was significantly higher with combined extract in comparison with individual extracts indicating greater inhibition of stress with combined extract than the individual extracts. Similarly, *W. somnifera* (100 mg/kg p.o.) treated group produced a statistically significant result by a reduction in glucose level, cholesterol level, triglyceride level, blood urea nitrogen level, and cortisol level as compared to control.[15]

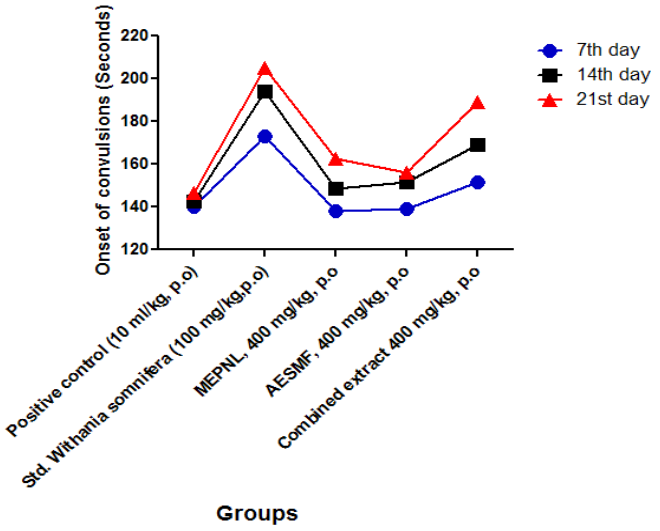


Fig. 4: Graphical representation of effect of MEPNL, AESMF and combined extract (MEPNL+AESMF) on anoxia stress tolerance test in mice.

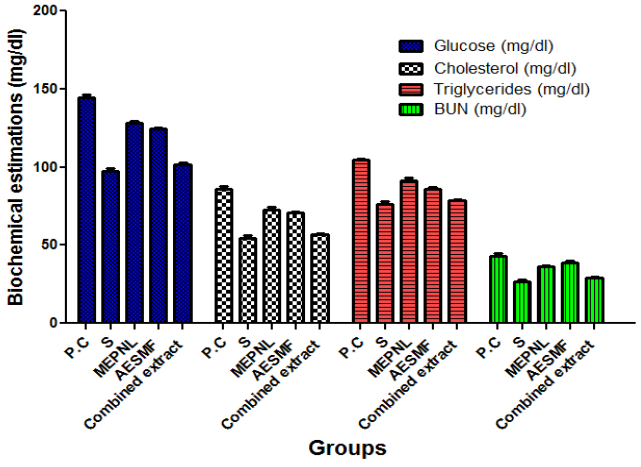
Table 2: Effect of MEPNL, AESMF & combined extract (MEPNL+AESMF) on anoxia stress tolerance test in mice

Duration of anoxia stress tolerance (Seconds)			
Groups	*7^{th} day*	*14^{th} day*	*21^{st} day*
Group I: Positive control (10 mL/kg, p.o)	140 ± 1.43	142.5 ± 1.59	146.83 ± 1.62
Group II: Standard (*W. somnifera* (100 mg/kg, p.o)	173 ± 1.68***	194.16 ± 1.53***	205 ± 0.83***
Group III: MEPNL (400 mg/kg, p.o)	138 ± 1.63	148.5 ± 0.93*	162.66 ± 0.67*
Group IV: AESMF (400 mg/kg, p.o)	139.3 ± 0.89	151.7 ± 0.64*	156.16 ± 1.73*
Group V: Combined extract (MEPNL+AESMF, 400 mg/kg, p.o)	151.7 ± 0.82*	169.12 ± 0.72**	189.17 ± 1.54***

Values are represented as mean ± SEM (n = 6), one way ANOVA continued by Dunnett test, *p < 0.05, **p < 0.01, ***p < 0.001 as equated to control.

Effects of methanolic extract of *P. niruri* leaves (MEPNL), aqueous extract of *S. mukorossi* fruit (AESMF) and combined extract (MEPNL+AESMF) on weight of the organs in immobilisation stress induced rats

As shown in above result Table 4, the combined extract (400 mg/kg orally) exhibited significant effect on organ weights than the individual extracts when equated to control. Similarly, *W. somnifera* (100 mg/kg orally) treated rats produced a statistically significant effect. The weight of the liver and adrenal gland was significantly decreased. The weight of the spleen and testes were significantly increased by combined extract compared to control group rats. Hence the combined extract was found to possess a remarkable organ protective effect than the individual extracts.



P.C = Positive control, S= Standard

Fig. 5: Graphical representation of the effect of MEPNL, AESMF and combined extract (MEPNL+AESMF) on biochemical parameters in immobilization stress-induced albino rats

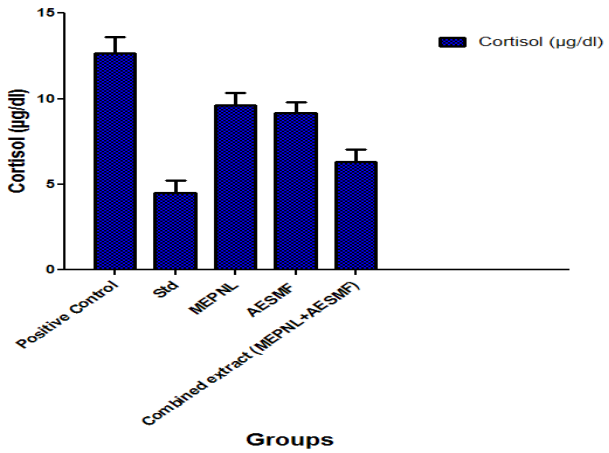


Fig. 6: Graphical representation of the effect of MEPNL, AESMF and combined extract (MEPNL+AESMF) on Cortisol levels (µg/dL) in an immobilization stress test.

Table 3: Effect of MEPNL, AESMF and combined extract (MEPNL+AESMF) on biochemical parameters in immobilization stress-induced albino rats

Biochemical estimations					
Groups	*Glucose (mg/dL)*	*Cholesterol (mg/dL)*	*Triglycerides (mg/dL)*	*BUN (mg/dL)*	*Cortisol (µg/dL)*
Group I: Positive control (10 mL/kg, p.o.)	144.5 ± 1.62	85.7 ± 1.72	104.38 ± 1.05	43.23 ± 1.82	12.66 ± 0.96
Group II: Standard (Withaniasomnifera (100 mg/kg, p.o.)	97.3 ± 1.98***	54.3 ± 1.72***	76.00 ± 1.95***	26.76 ± 1.04***	4.5 ± 0.72***
Group III: MEPNL (400 mg/kg, p.o)	128 ± 1.43*	72.4 ± 1.85*	91.2 ± 1.73*	36.12 ± 0.83*	9.62 ± 0.73*
Group IV: AESMF (400 mg/kg, p.o)	124.3 ± 0.71*	70.6 ± 0.74*	85.6 ± 1.45**	38.53 ± 1.63*	9.16 ± 0.64*
Group V: Combined extract (MEPNL+AESMF, 400 mg/kg, p.o)	101.7 ± 0.82***	56.8 ± 0.81***	78.6 ± 0.94***	29.18 ± 0.56***	6.3 ± 0.71**

Values are represented as mean ± SEM (n = 6), one way ANOVA continued by Dunnett test, *p < 0.05, **p < 0.01, ***p < 0.001 as equated to control.

Table 4: Effect of MEPNL, AESMF & combined extract (MEPNL+AESMF) on weights of organs in Immobilisation stress induced rats

Organ weight gm/100 gmb.w				
Groups	*Liver*	*Adrenal gland*	*Spleen*	*Testes*
Group I: Positive control (10 mL/kg, p.o)	5.495 ± 0.10	0.035 ± 0.94	0.249 ± 0.05	1.264 ± 0.001
Group II: Standard (*W. somnifera* (100 mg/kg, p.o)	3.635 ± 0.09***	0.016 ± 0.04***	0.361 ± 0.041***	1.563 ± 0.005***
Group III: MEPNL (400 mg/kg, p.o)	4.512 ± 0.82*	0.029 ± 0.01*	0.312 ± 0.732*	1.32 ± 0.007*
Group IV: AESMF (400 mg/kg, p.o)	4.685 ± 0.94*	0.025 ± 0.03*	0.281 ± 0.843*	1.37 ± 0.006*
Group V: Combined extract (MEPNL+AESMF, 400 mg/kg, p.o)	3.832 ± 0.85***	0.019 ± 0.06**	0.357 ± 0.749***	1.541 ± 0.003***

Values are represented as mean ± SEM (n = 6), one-way ANOVA continued by Dunnett test, *p < 0.05, **P<0.01, ***P<0.001 as equated to control.

Table 5: Effect of MEPNL, AESMF and combined extract (MEPNL+AESMF) on swimming endurance test in Mice

Swimming endurance test			
Groups	*Species*	*No. of animals*	*Swimming time (minutes)*
Group I: Positive control (10 mL/kg, p.o)	Swiss albino mice	6	23.66 ± 1.82
Group II: Standard (W. somnifera (100 mg/kg, p.o)	Swiss albino mice	6	47.8 ± 0.91***
Group III: MEPNL (400 mg/kg, p.o)	Swiss albino mice	6	36.7 ± 0.67*
Group IV: AESMF (400 mg/kg, p.o)	Swiss albino mice	6	38.1 ± 1.83*
Group V: Combined extract (MEPNL+AESMF, 400 mg/kg, p.o)	Swiss albino mice	6	45.3 ± 1.54***

Values are represented as mean ± SEM (n=6), one way ANOVA continued by Dunnett test, *P < 0.05, **P < 0.01, ***P<0.001 as equated to control.

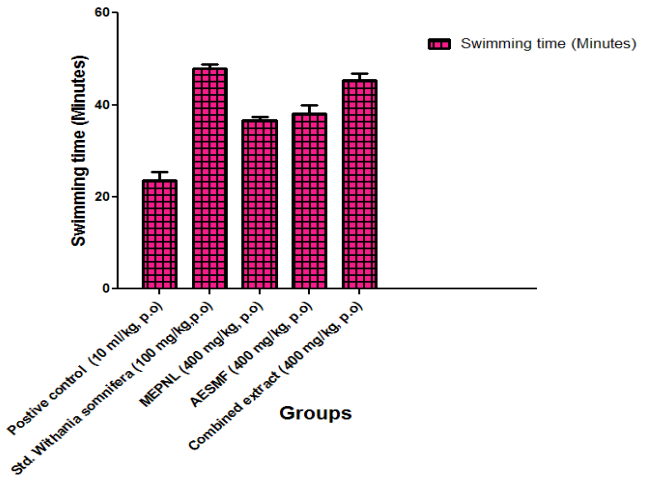


Fig. 7: Graphical representation of effect of MEPNL, AESMF & combined extract (MEPNL+AESMF) on swimming time in swimming endurance test in mice.

Influence of Methanolic Extract of *P. niruri* leaves (MEPNL), Aqueous Extract of *S. mukorossi* Fruit (AESMF), and Combined Extract (MEPNL+AESMF) on Swimming Endurance Test in Mice

As shown in the above result Table 5 and Fig. 7, combined extract (MEPNL+AESMF) (400 mg/kg orally) exhibited a significant anti-stress effect than the individual extracts by a rise in the swimming time when compared to positive control group. Similarly, standard *W. somnifera* (100 mg/kg orally) treated mice produced a statistically significant result by an increase in swimming time as compared to control.

Discussion

In the present study, anti-stress activity was evaluated in several experimental models. The investigation reports showed that the anti-stress activity of combined extract of P. niruri leaves and S. mukorossi fruit was highly significant compared to the individual extracts.[16] In anoxia stress, the onset of convulsion time was taken as a parameter to assess the degree of stress. There was an increase in the onset of convulsion time in the combined extract of P. niruri leaves and S. mukorossi fruits administered group (400 mg/kg orally) on 7th, 14th, and 21st day when compared to individual extracts, thus confirming its anti-stress and anti-oxidant properties.[17] The mechanism involved in an increase in serum cholesterol by stress is attributed to the enhanced response of the hypothalami-hypophyseal axis (HPA), resulting in the release of catecholamines and corticosteroids. The increase in release of catecholamines leads to an elevated level of glucose and blood urea nitrogen. Stress stimulates adreno-medullary activity in man. In turn, epinephrine activates pituitary glandular β2 receptors causing a greater release of cortisol. In the immobilization stress model MEPNL, AESMF, and combined extract (400 mg/kg orally) decreased the elevated levels of serum biochemical parameters such as serum glucose level, cholesterol level, triglyceride level, blood urea nitrogen level, and cortisol level, thus confirming its anti-stress activity.[18] However, the combined extract has reduced the biochemical parameters to a greater extent than the individual extracts indicating the synergistic effect with the combined extract. Cortisol enhances mRNA levels in hepatocytes. This results in an increase in liver weight. Constriction of the spleen during stress causes release of more blood cells (RBC). Thereby the spleen weight decreases during stress. Stress-induced modifications in the weight of the organs were significantly reversed by the test extracts (MEPNL, AESMF and combined extract).[19]

The swimming endurance model is the most widely applicable technique for evaluating the anti-stress property of a novel preparation. In the swim endurance test, it is well validated that drugs with anti-stress activity increase the swimming time. The investigations carried so far indicate that combined extract (MEPNL+AESMF) had a protective effect on the experimental animals against the alterations imposed due to the swimming endurance test.[20]

Experimental investigations have established the adaptogenic properties of *P. niruri* leaves and *S. mukorossi* fruits. The effects are plausibly due to the presence of flavonoids and saponins present in the plants.[21]

Literature review signifies that flavonoids and saponins were reported to have a variety of pharmacological activities, including anti-stress activity.[22] In the present research, preliminary phytochemical screening on MEPNL and AESMF displayed positive tests for flavonoids and

saponins; this could be the rationale for a significant adaptogenic property of test extract.[23]

Conclusion

In the present research, methanolic extract of *P. niruri* leaves (MEPNL), aqueous extract of *S. mukorossi* fruit (AESMF), and combined extract (MEPNL+AESMF)has been evaluated for anti-stress ("Adaptogenic") activity by inducing various stressful situations in animals. Our present study confirmed that the combined extract (MEPNL+AESMF)has significant anti-stress activity than the individual extracts of *P. niruri* leaves and *S. mukorossi* fruit in all the animal models exhibiting synergistic anti-stress potential with the combined extract. It can be hypothesized that combined extract of *P. niruri* leaves and *S. mukorossi* fruit may upregulate the norepinephrine, serotonin, and dopamine levels in the brain or it may be involved in the inhibition of monoamine oxidizes, or the extract can cause sensitization of serotonin receptors that results in mood elevation.

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