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Combined And Comparative Biochemical and Behavioral Assessment of Delphinium Denudatum and Amaranthus Spinosus for Anti-Stress, Nootropic and Antioxidant Activities

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Abstract

Background: Stress defined as an imprecise reaction of the body to any kind of stimuli on it and disturbs normal physiological condition, threatened homeostasis. Stress causes decline in health by disturbing behaviour, physical and hormonal system of the body.
Methods: The test drugs Dephinium denudatum root and Amaranthus spinoses leaves were defatted with petroleum ether (60-80 ^{0}C) and then extracted with hydroalcohlic solvent (Ethanol 95%, v/v: water, 1:1) by soxhlation process. The hydroalcohlic extract of both the drugs singly and in combinations was evaluated for experimental activity in Wistar albino rats in the doses of 200 and 400 mg/kg by using different anti-stress tests like Swimming endurance and post swimming muscle coordination test (physical stress), Immobilization stress test and Anoxia stress tolerance test, antioxidant activity by DPPH, Reducing power methods, Nitric oxide scavenging activity, and H_2O_2 assay method and nootropic activity was done by Elevated plus maze test, Morris water maze test and estimation of Acetylcholine esterase level.
Results: In the dose dependent manner, both the hydroalcohlic extracts and combination of the higher doses produced the anti-stress activity, antioxidant and nootropic activities.
Conclusion: Hydroalcohlic extracts of Dephinium denudatum root, Amaranthus spinoses leaves and combination of both the drugs may act as anti-stress, antioxidant and nootropic agents in rats. Amaranthus spinoses was found to be more effective compare to the Dephinium denudatum.

Keywords: Delphinium denudatum, Amaranthus spinosus, Stress, Antioxidant, Nootropic etc.

INTRODUCTION

The quality of human life is influenced by present advancement in science and technology. However, modern life pattern, responsibilities, difficulties, workload and related factors responsible for development of mental diseases like depression, insomnia, amnesia, anxiety, epilepsy, stress, Alzheimer's**,** parkinsonism, schizophrenia, migraine etc. Mental diseases deal to 12.3% of the worldwide problem and might be reached up to 15% by 2020 (Reynolds 2003) [1].

Stress defined as an imprecise reaction of the body to any kind of stimuli on it and disturbs normal physiological condition, threatened homeostasis [2]. Stress causes decline in health by disturbing immune and hormonal system of the body [3]. Stress causes the production of free radicals which can lead to the dementia. It is reported that stress methods have shown that mental (dementia) and physical tension stimulates the hypothalamic-pituitary-adrenal axis and sympathetic nervous system, causes the release of adrenaline, noradrenaline and glucocorticoids from the adrenal gland [4, 5]. Glucocorticoid secretion in excess amount might lead to the development of various diseases like hypertension, ulcers, immunosuppressant, reproductive impairment and diabetes etc [6, 7].

There are number of natural drugs that are using for different types of mental disorders, getting hold of increasing popularity over other types of system of medicine related to the number of diseases [8, 9]. Natural drugs found to be having significantly minor harmful effect to that of synthetic drugs though having equivalent efficiency. Now a day, plant products are gaining popularity in many section of the population [10].

Delphinium denudatum/Ranunculaceae (black) is found to be effective in a variety of disorders like fungal infections, dysurea, calculi, asthma, cough, jaundice and nervous problems [11, 12]. In Unani and Ayurveda system of medicine root is used in a variety of remedial formulations to diminish the withdrawal symptoms in people of de-addiction therapy [13] Khameera Gaozaban Ambari Jadwar Ood Saleeb Wala, Habb-e Jadwar, Habb-e Jawahar and Jawahar Mohra, Marham-e Jadwar, Zimad-e Warm-e Lozatain are some of the formulations of Jadawar in Unani System of Medicine for CNS disorders [14]. Its claims many bioactive constituent, some of which are diterpenoid, triterpenoid, flavanoids, and different types of alkaloids [15].

Amaranthus spinosus Linn. is an erect, spinous annual or perennial herb is widely distributed in most of the regions of Asia. Amaranthus spinosus reported to be used as febrifuge, antipyretic, diuretic, laxative digestible, bronchitis, leprosy, epilepsy, piles, hallucination, healing of wounds and rheumatism. The whole plant is reported to contain number of bioactive constituents [16, 17].

In the Ayurvedic and Unani system of medicine, combined extracts of plants & herbal formulation are used as drug of choice rather than single drug and there is a lack of scientific data related to antis-tress, nootropic and antioxidant activities for these plants. In this context, the present study has been designed to carry out combined and comparative studies of Dephinium denudatum root and Amaranthus spinoses leaves for anti-stress, antioxidant and nootropic activities.

MATERIALS AND METHODS

Chemicals:

Tween 80 and Petrolium ether obtain from SD fine chemicals, Withania Somnifera (Dabur India Ltd, Baddi, HP, India) and Chloroform (Nice chemicals, Cochin, India) and Kits for all biochemical evaluation testwere procured from Transasia biomedical Ltd. Baddi (HP) India.

All the chemicals used in the study were of analytical grade.

Preparation of extract:

Plant material like root of Delphenium denudatum were collected from local market of Moradabad Uttar Pradesh and leaves of Amaranthus spinosus from IFTM university botanical garden. Amaranthus spinosus leaves were authenticated by the Botanist, Dr. Beena Kumari, Hindu College, Moradabad. A plant specimen (Voucher No. HC.MBD/HAP/BK/2016/01/488) was submitted in the herbarium, and Delphenium denudatum root was authenticated by Dr. Ashok Kumar, IFTM University, Moradabad Uttar pradesh and plant specimen was kept in the herbarium with the Voucher No. 2015/SOS/BOT/14. The botanical taxonomy of the plants was properly matched with standard floras and also cross-checked with Herbarium files. The drugs were made into coarsely powdered then subjected to the extraction with petroleum ether (60-80n^{o}C) followed by with hydro-ethanolic mixture (Ethanol 95%, v/v: water, 1:1) in a Soxhlet apparatus. The extracts were filtered and distilling off the solvents separately and evaporated to dryness using rotatory vacuum evaporator.

Animals

The recent experimental work was done on either sex of Wistar albino rats weighing (120-180g). Animals

were procured from the animal house of the I.F.T.M. University, Moradabad and maintained on a natural day–night cycle (12hr dark: 12hrs light) at room temperature of about 24-26°C, with free access to standard food pellets and water ad libitum. Before exposure to experiment, animals were acclimatized for at least ten days. Experiments were carried out between 10:00am-5:00pm. The study was approved by the Institute Animal Ethics Committee, Department of Pharmacology and Clinical Research, College of Pharmacy, IFTM University, Moradabad. All animal care and experimental protocols were in compliance with the National Institute of Health (NIH) guidelines for the care and use of the laboratory animals (NIH, 1985). The animals in each group contained six animals (n=6).

Selection of the doses of the test drugs

Both the doses (200 and 400mg/kg) of the test drugs were used for recent work [18].

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Anti stress activity

Swimming endurance and post swimming muscle coordination test (physical stress):

On 7th day, one hour after drug administration, the each rat was endorsed to swim inside a perplex glass container (30 cm height with 20 cm diameter, containing water up to 25 cm height) maintained at 26 ± 1ºC. The end point was considered when rats were become motion less (exhausted) [19, 20]. The animals were taken out and let them pick up and dry for about 5 min. the animals were then put on rotating rod rotating at 15 rpm time to stay on the rotating rod was noticed [21]. The animals were divided into 8th groups and every group consist of 6 rats. Group I- control group, received vehicle (5 ml/kg p.o), group II and III- received Delphenium denudatum extract (DDE) (200 and 400mg/kg, p.o.) group IV and V received Amaranthus spinosus extract (ASE) (200 and 400mg/kg, p.o.) groups VI and VII received combination of both the drugs (50 and 100 mg/kg, p.o. of each drugs) and group VIII was received the standard drug Withania somnifera (100 mg/kg, p.o). The standard drugs and tests drugs were administered to the animals once every day for seventh (7th) days.

Immobilization stress test (IST)

In the Immobilization stress model; the animals were divided into nine groups and each group containing six rats. Group I- control group, received vehicle (5 ml/kg p.o), group II is the stress control group, group III and IV received DDE (200 and 400mg/kg p.o) group V and VI received ASE (200 and 400mg/kg p.o) groups VII and VIII received combination of both the drugs in 50 and 100 mg/kg, p.o., of each drugs and group IX is received the standard drug (WS 100 mg/kg, p.o). The standard drugs and tests drugs were administered to the animals once every day for seventh (7th) days.

The stress was given by restraining the animals inside an adjustable acrylic hemi-cylindrical plastic tube (4.5 cm diameter, 12 cm long). The rats were restricted alone for a period of 150 minutes once daily for 7th successive days. On last day of experiment, straight away after the last experience to stress, blood was collected from retro-orbital plexus under light ether anaesthesia and serum were taken for biochemical estimation after that animals were sacrificed and the weights of organs were reported [22].

Anoxia stress tolerance test (ASTT)

In this test, animals were undergone to hypoxic condition. One hour after drugs administration, stress was induced in the animals by keeping each animal individually in a hermetic vessel of 500 ml capacity to note hypoxia tolerance time. The animals at once removed when the first convulsions appeared. The time duration of anoxia tolerance was noted [23]. The animals were divided into eight groups and every group contain 6 rats. Group I- control group, received vehicle (5 ml/kg p.o), group II and III- received DDE (200 and 400mg/kg, p.o) group IV and V received ASE (200 and 400mg/kg, p.o.) groups VI and VII received combination of both the drugs (50 and 100 mg/kg, p.o. of each drugs) and group VIII was received the standard drug Withania somnifera (100 mg/kg, p.o). The standard drugs and tests drugs were administered to the animals once every day for seventh (7th) days.

In vitro Antioxidant activity

DPPH radical scavenging assay

The free radical scavenging limit of the hydroethanolic extract of Amaranthus spinosus (ASE) and Delphinium denudatum (DDE) were evaluated by DPPH method. DPPH (200µM) arrangement was set up in 95% methanol. From the stock plant separate arrangement 25, 50, 75, 100, 125 and 150 µg/ml were taken in six test tubes. 0.5ml of newly arranged DPPH arrangement was incubate with test drug and following 10 minutes, absorbance was taken as 517 nm utilizing spectrophotometer. Standard ascorbic acid was utilized as reference. Count % searching of the DPPH free radical was estimated utilizing following equation [24].

Scavenging impact (%) = [1-Abs. of test/Abs. of control] x 100

Reducing power assay

1 ml of different concentration (1-5 mg/ml) of test drugs extract was blended with 2.5 ml phosphate buffer and 2.5 ml of potassium ferricyanide. The blend was incubated at 50°C for 20 min. Aliquots of 2.5 ml of trichloroacetic corrosive were added to the blend, which was then centrifuged at 3000 rpm for 10min. The upper layer of the arrangement (2.5 ml) was blended with break even with volume of distil water, to this 0.5ml of naturally arranged ferric chloride arrangement was included and the absorbance was estimated at 700 nm. Increased absorbance of the response blend demonstrates increased in reducing power [24].

% of inhibition = Control – test x 100/control

Nitric oxide scavenging activity

Sodium nitroprusside (5 mM) in standard phosphate support preparation was incubated with various fixations (20-150µg/ml) of the ethanolic plant extracts, dissolved in phosphate buffer (0.025 M, pH 7.4), and tubes were incubated at 25 ^{o}C for 5 hours. The control tube without the plant extract, however, with a comparable measure of buffer, was kept up in an identical way. 0.5ml of the incubated solution was evacuated and diluted with 0.5ml of Griess reagent (1% sulfanilic acid, 5% phosphoric acid and 0.1%) after 5 hours. The absorbance of the chromophore form during diazotization of nitrite particles with suphanilic acid and its consequent coupling with napthylethylenediamine was read at 546 nm. The experiment was repeated in triplicate [25].

Scavenging effect (%) = [1-Abs. of test/Abs. of control] x 100

H_2O_2 radical scavenging activity

The H2O2 scavenging capacity of ethanolic concentrate of ASE and DDE was determined [24]. Counteractive action of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogens, 10, pp. 1003-1008. 0.2-1.0 ml of test (10 mg/10 ml) was taken in various test tubes, to which 1 ml of H2O2 was added. The tubes were incubated for 5 minutes at room temperature. After 5 minutes, 2 ml of potassium dichromate acetic reagent was included and the tubes were incubated for 10 minutes at room temperature. The absorbance estimation of the reaction mixture was recorded at 700 nm. A blank containing the phosphate buffer without the plant extract and a standard was calculated as

% searching [H2O2] = [A control: A test/A control] ×100

Where ‘A control’ was the absorbance of the control, and ‘A sample’ was the absorbance of the sample.

Nootropic activity

Elevated plus maze (EPM)

The EPM as the exteroceptive behavioural model to assess learning and memory in animals. The animals were divided into nine groups and each group consists of six rats. Group I is the control group received vehicle (5 ml/kg p.o), Group II treated with Scopolamine (1 mg/kg, i.p.), group III and IV received DDE 200 and 400mg/kg, p.o., group V and VI received ASE 200 and 400mg/kg, p.o., groups VII and VIII received combination of both the drugs in 50 and 100 mg/kg, p.o., of each drugs and group IX is received the standard drug (Vit C 250 mg/kg, p.o). The tests drugs and standard drug was given to the rats once daily for 7th days. On the 7thday, each group was treated with Scopolamine (1 mg/kg, i.p.) except control group I hr prior to the test. Transfer latency (TL) was taken as the time taken by the rats to move into any of the secured arms with all itsfour legs. TL was noticed on the 7th day for 5 min. The rats were permitted to investigate the maze for 10 sec and after that came back to its home cage. Memory retention was analyzed 24 hr after the 7th day trial on the 8th day [26]. The Inflexion ratio (IR) utilizing the equation, IR = (L1 – L0)/L0 Where, L0 = TL on day-2/day-9 in sec. L1
= initial TL in sec.

The Morris water maze (MWM)

The MWM comprises of substantial round tank made of dark cloudy PVC or hard board covered with fiberglass and resin and after that surface painted white (1.8-2.0m in distance across and 0.4-0.6m stature). The pool is loaded up with water (20-22^{0}C) to a depth of 0.3-0.4m, and rendered opaque by the expansion of little amount of drain or non-poisonous white shading. The pool is settled with filling and depleting offices and mounted on a casing so the water is at abdomen level. The floor of round tank is separated in to four equivalent quadrants subjectively planned north, south, east or west. Furthermore, escape stage is made of Plexiglas with a13 cm square stage appended to a 34 cm long clear Plexiglas round and hollow platform (3cm. distance across) mounted on a 1sq. m (5mm thick) Plexiglas base. The highest point of the stage is secured with a coarse material that gives a decent grasp to the rodent when jumping on a stage. For the concealed stage

errand, water is added to round tank to a level 2cm over the highest point of the stage. Water maze represents the versatile tools in which various particular assignments can be estimated. The least difficult proportion of execution is the inertness to escape from the water on to the concealed stage. Time to locate the shrouded stage is considered as escape latency (EL). The stage in the water maze was kept at a similar position all through the test to survey the impact of medications on spatial reference memory. Grouping and treatment was same as above. The tests drugs and standard drug was given to the rats once daily for 7^{th} days [27]. The ‘‘inflexion ratio (IR)’’ using the formula, IR = (L1 – L0)/L0 Where, L0 = transfer latency on day-2/day-9 in sec. L1 = initial transfer latency in sec.

Estimation Acetyl cholinesterase

Estimation of Acetylcholinesterase (AChE) on the seventh day, all animals were immediately sacrificed, beheaded and brain was detached from the skull quickly. The entire brain AChE inhibitory action of test drugs was estimated. It is suggested that the prefrontal cortex, hippocampus and hypothalamus are imperative districts of mind engaged with preparing of memory and they are rich in cholinergic neurons. The rate of colour development is the proportion of the AChE movement. Change in absorbance every moment of the example was read at 412 nm. The compound movement is communicated as the 'n' moles of substrate hydrolyzed/min/mg of protein. The protein substance in the brain test homogenates were resolved utilizing Ellman Lowry technique [28]. Treatment and grouping is same as EPMT.

Statistical analysis

The result calculated as Mean ± SEM. The experimental groups were compared by one-way Analysis of Variance (ANOVA) followed by Dunnett’s test and was considered statistically significant when $p<0.05$.

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RESULTS

In the recent work it was found that ASE (200 mg/kg) and Combination C_1 showed significant ($p<0.05$) effect, DDE and ASE ((400 mg/kg)) Withania somnifera (WS) (100mg/kg) and Combination C_2 (100 mg/kg) more significantly ($p<0.01$) increased in the swimming endurance time and duration of stay on rota rod as the results are revealed below.

Table 1: The effects of DDE, ASE, C_1, C_2 and WS on stress performance by swimming endurance model and anti-fatigue test

S. N.	Groups	Dose (mg kg^{-1}, orally)	SE time in minutes (in sec)	Post swimming antifatigue test by rota rod (in sec)
1	**Control (vehicle)**	5 ml	86.43±23.26	24.54±13.65
2	**DDE**	200	133.43±18.44*	$28.45±34.45^{ns}$
3	**DDE**	400	143.52±36.47**	35.75±23.45*
4	**ASE**	200	112±38.52*	38.45±12.05*
5	**ASE**	400	164.54±21.74**	47.82±15.08**
6	**Combination C_1**	50	106±38.52*	38.45±12.05*
7	**Combination C_2**	100	184.42±17.56**	58.82±15.08**
8	**WS**	100	177.33±43.03**	47.12±06.45**

Each value represents the Mean ± SEM, (n=6), ns= non-significant, *p<0.05, **p<0.01, ***p<0.001comparedwith the control group (ANOVA followed by Dunnett’s test.

The experimental model of immobilization stress trial (IST) on biological parameters indicated that, DDE (200-400 mg/kg) significantly reduced level of glucose and cholesterol, ASE (200-400 mg/kg), Combination (C_1 and C_2) and standard drug Withania sominefera (WS) all were more significantly reduced the glucose and cholesterol level, DDE (400 mg/kg) and ASE (200-400 mg/kg) significantly reduced level of triglyceride (TG) where as DDE (200 mg/kg), Combinations (C_1 and C_2) and standard drug WS all were more significantly reduced the triglyceride level, DDE (200-400 mg/kg), ASE (200-400 mg/kg), Combinations (C_1 and C_2) and standard drug WS all were more significantly reduced the level of blood urea nitrogen (BUN) compare to

stress control group as the results are shown in table 5.18. Related to hematological parameter it was observed that, DDE (200-400 mg/kg) and ASE (200mg/kg) produced significant effect whereas ASE (400mg/kg), Combinations (C_1 and C_2) and standard drug WS all were more significantly reduced the WBC and RBC count compare to stress control group as the results are shown in table 2.

Table 2. The effects of DDE, ASE, C_1, C_2 and standard drug WS on biochemical and hematological parameters in immobilization stress test (IST)

Treatment Groups (mg/kg p.o)	Biological parameters (mg/dl)				Hematological parameters	
	Glucose(µm/dl)	Chol. (µm/dl)	TG (µm/dl)	BUN (µm/dl)	Total WBC /10^3	Total RBCm/10^3
Control (vehicle-5ml/kg)	76.40±5.814	59.18±2.71	60.56±2.60	44.77±6.07	7454±314	4.24±0.21
Stressed control (SC) (vehicle-5ml/kg)	141.10±6.45[a]	78.59±4.34[a]	82.13±2.26[a]	116.0±4.07[a]	11563±114[a]	6.14±0.05[a]
DDE 200	108.57±8.00*	54.04±4.41*	62.71±5.28*	54.01±2.02**	9124±110*	5.16±0.15*
DDE 400	105.96±8.82*	54.00±4.55*	56.75±11.40*	58.71±4.31**	8719±213*	5.18±1.05*
ASE 200	76.57±3.50**	52.14±6.31**	59.51±15.48*	51.01±2.02**	9124±110*	5.16±0.15*
ASE 400	75.96±6.32**	46.92±4.55**	56.45±4.20*	45.65±4.81**	8319±213**	5.08±1.05**
Combination C_1 50	83.95±13.20***	54.14±6.31*	49.51±15.48**	49.01±12.05**	7100±110**	5.06±0.15**
Combination $C_2$100	68.96±6.32**	44.92±4.75**	41.45±4.20**	41.65±4.81**	7119±213**	5.01±1.05**
WS 100	70.55±7.94**	48.60±3.16**	54.08±7.61**	56.00±7.33**	7981±213**	4.26±0.05**

Each value represents the Mean ± SEM, (n=6), ns= non-significant, [a]p<0.05 compared to the control, *p<0.05, **p<0.01, ***p<0.001, (ANOVA followed by Dunnett's against with the stress control).

In immobilization stress induced changes in organ weight test, weight of liver and adrenal gland was increased, while weight of spleen and testis was reduced in stressed group compared to unstressed group. Pretreatment with DDE and ASE (200mg/kg), Combination C_1 (50 mg/kg each drug) produced significant effects while ASE and DDE, Combination C_2 (100 mg/kg each drug) and WS (100 mg/kg) more significantly weight of liver and adrenal gland was decreased, while weight of spleen and testis was increased compared to stressed control group as the results are shown in table 3.

Table 3. The effects of DDE, ASE, C_1, C_2 and standard drug WS on immobilization stress induced changes in organ weight

Treatment groups (mg/kg, p.o)	Organ weight (g)			
	Liver	Adrenal gland	Testes	Spleen
Unstressed control, vehicle (5ml/kg)	0.67±0.02	0.025±.006	0.46±0.04	0.36±0.07
Stressed control vehicle (5ml/kg)	0.93±0.23[a]	0.039±.005[a]	0.29±0.03[a]	0.19±0.04[a]
DDE 200	0.87±0.08*	0.036±.002*	0.33±0.003*	0.23±0.01*
DDE 400	0.83±0.09**	0.029±.01**	0.37±0.09**	0.27±0.04**
ASE 200	0.75±0.05*	0.033±.0016*	0.34±0.01*	0.27±0.02**
ASE 400	0.69±0.03**	0.027±.02**	0.39±0.09**	0.29±0.06**
Combination C_1 50	0.79±0.09*	0.035±.002*	0.34±1.01*	0.22±0.09*
Combination $C_2$100	0.65±0.02**	0.026±.002**	0.41±0.001**	0.31±0.11**
WS 100	0.78±0.03**	0.033±.008*	0.40±0.07**	0.30±0.06**

Each value represents the Mean ± SEM, (n=6), ns= non-significant, [a]p<0.05 compared to the control, *p<0.05, **p<0.01, ***p<0.001, (ANOVA followed by Dunnett's against with the stress control)

Hypoxia stress tolerance test

In this test, it was observed that, DDE (200 mg/kg) produced significant effect whereas DDE (400mg/kg), ASE (200-400mg/kg), Combination (C_1 and C_2) and standard drug WS (100 mg/kg) all were more significantly increased the hypoxia stress tolerance compare to the control group as the results are shown in table 4.

Table 4. The effects of DDE, ASE, C_1, C_2 and standard drug WS on hypoxia stress tolerance test (HSTT) in rats.

S.N.	Treatments Groups	Duration of hypoxia stress tolerance in minutes
1	Control (Vehicle 5 ml/kg)	126.17±4.84
2	DDE (200 mg/kg)	195.36±3.106*
3	DDE (400 mg/kg)	209.33±5.20**
4	ASE (200 mg/kg)	205.36±3.106**
5	ASE (400 mg/kg)	210.43±5.32**
6	Combination C_1 (50 mg/kg)	180.36±3.106**
7	Combination C_2 (100 mg/kg)	218.43±5.32**
8	WS 100 mg/kg	213.10±5.68**

Results are mean ± SEM (n = 6). ns= non-significant, *P < 0.05 and **P < 0.01, compared to the control, one-way ANOVA with Dunnett's test.

Several concentrations ranging from 25–150 µg/ml of the ASE, DDE, and combined C extract were tested for their antioxidant activity in different in vitro models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all models given below in tables 5, 6, 7, and 8. Antioxidant power is indirectly proportional to the IC_{50} value. The lower the IC_{50} value, the greater the antioxidant activity. In this test, combined C extract showed comparable response to the standard drug. The standard drug ascorbic acid showed more antioxidant activity compared to the ASE and DDE. The ASE was found to be better than the DDE. In some models of antioxidant activity, as shown below, ASE produced a comparable effect to that of the standard ascorbic acid.

Table 5. DPPH scavenging activity of ASE, DDE, combined C and ascorbic acid

Test	Concentration (µ/ml)	% Inhibition of ASE extract	% Inhibition of DDE extract	% Inhibition of Comb. C extract	% Inhibition of Ascorbic acid
DPPH (2,2-Diphenyl-1-picryl hydrazyl)	25	25	20.14	25.45	29.01
	50	29.66	24.40	31.66	45.94
	75	41.60	38.24	45.60	55.58
	100	50.34	48.31	52.34	65.81
	125	78.80	64.45	78.80	71.23
	150	81.6	75.35	85.6	87.34
IC_{50}		135	151	129	124

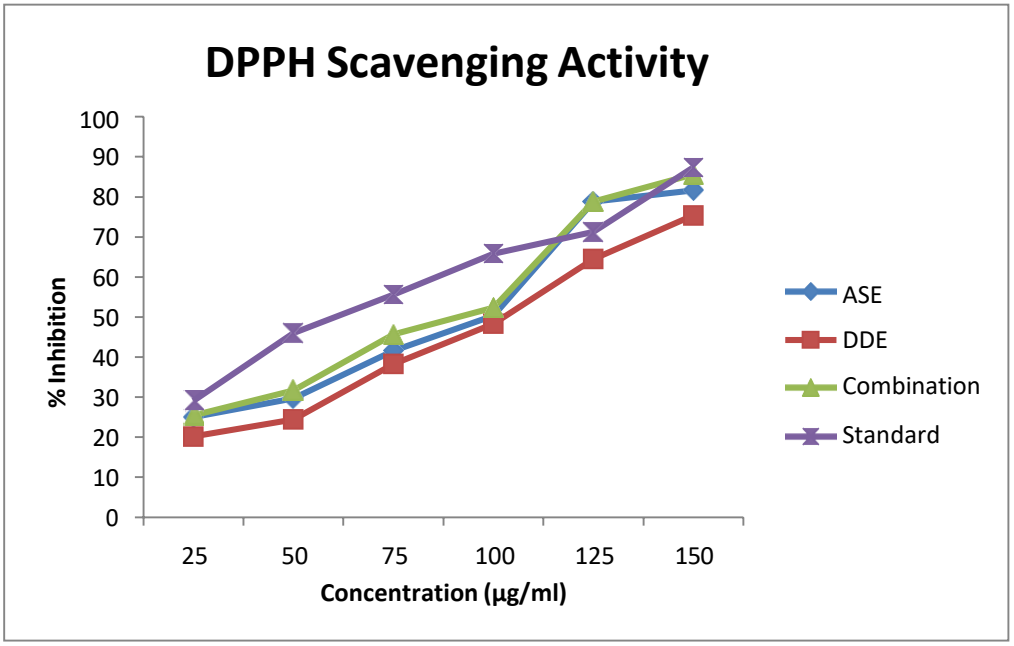

Figure 1: Effect of DDE and ASE and ascorbic acid in DPPH method

Table 6. Reducing power activity of ASE, DDE, combined C and ascorbic acid

Test	Concentration (µ/ml)	% Inhibition of ASE extract	% Inhibition DDE of Extract	% Inhibition of Comb. C extract	% Inhibition of Ascorbic acid
Reducing power	25	41.25	39.54	44.26	58.53
	50	52.30	43.52	56.60	66.12
	75	60.23	54.23	69.28	72.68
	100	70.34	64.58	76.34	79.91
	125	85.80	71.56	85.10	85.65
	150	91.62	79.35	92.62	95.34

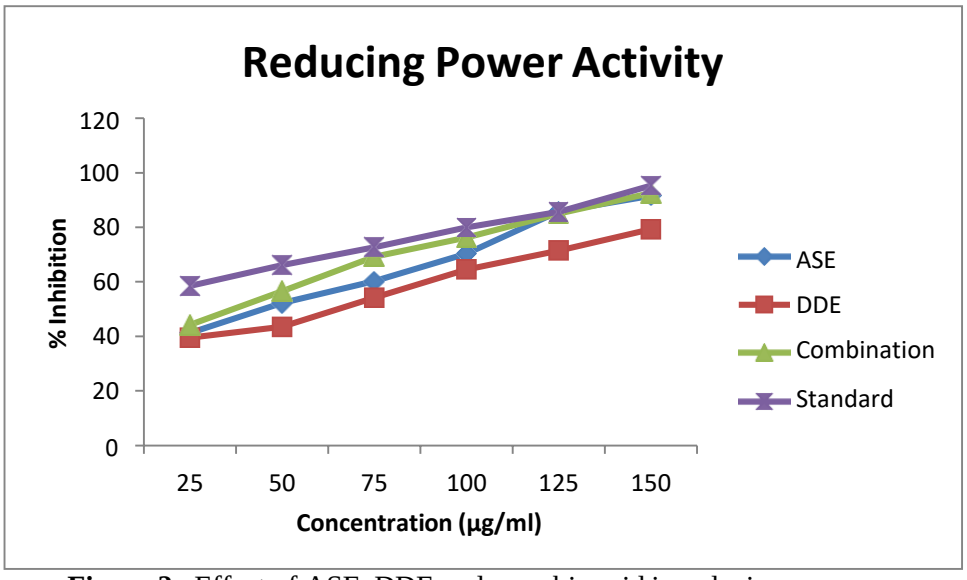

Figure 2. Effect of ASE, DDE and ascorbic acid in reducing power.

Table 7. Nitric oxide scavenging activity of of ASE, DDE, combined C and ascorbic acid

Test	Concentration (µ/ml)	% Inhibition of ASE Extract	% Inhibition DDE of Extract	% Inhibition of Comb. C extract	% Inhibition of standard
Nitric oxide	25	23.34	21.35	24.32	26.45
	50	32.75	30.56	33.05	34.25
	75	42.59	39.36	44.19	46.78
	100	54.76	50.26	54.76	56.47
	125	63.58	59.43	65.58	67.24
	150	73.62	67.45	75.62	76.98
IC_{50}		153	170	149	142

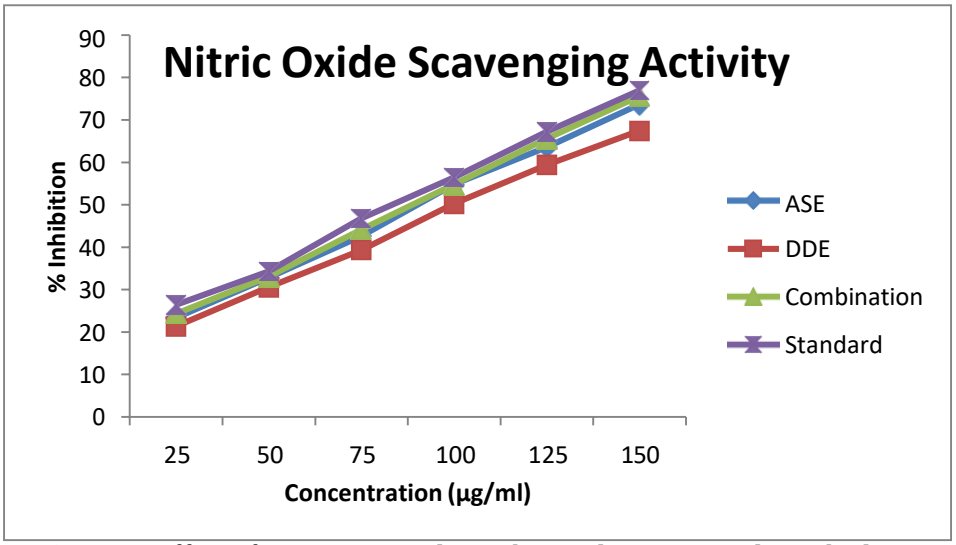

Figure 3. Effect of ASE, DDE and ascorbic acid in nitric oxide method.

Table 8. H_2O_2 Assay of ASE, DDE, combined C and ascorbic acid

Test	Concentration (µ/ml)	% Inhibition of ASE Extract	% Inhibition DDE of Extract	% Inhibition of Comb. C extract	% Inhibition of standard
H_2O_2 Assay	25	23.76	20	23.79	24.64
	50	31.36	21.56	34.96	34.28
	75	48.26	29.35	48.22	46.21
	100	56.18	39.46	56.18	55.47
	125	61.38	56.45	62.38	62.45
	150	70.64	68.25	73.64	72.65
IC_{50}		157	175	152	155

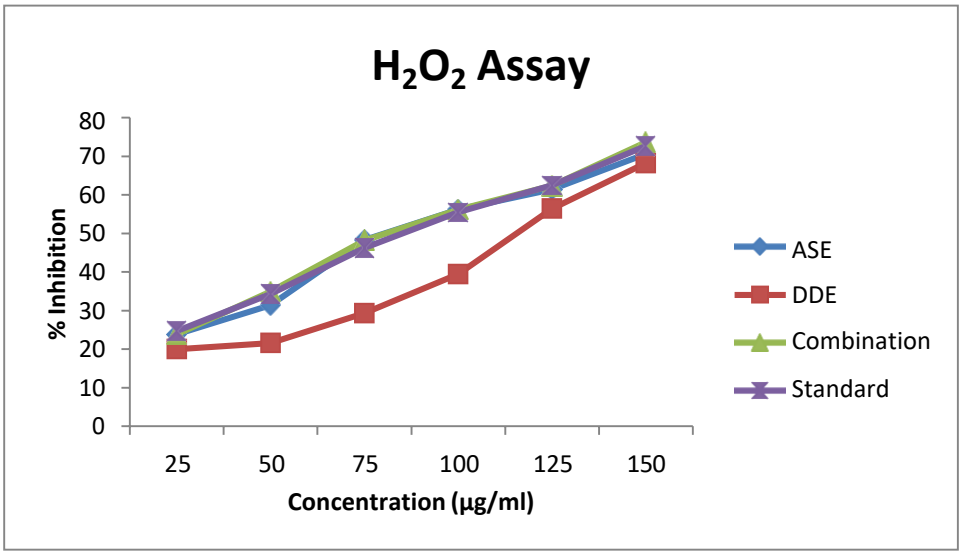

Figure 4. Effect of ASE, DDE and ascorbic acid in H_2O_2 Assay.

In this test, DDE (200 mg/kg) and Combination C1 (50 mg/kg each drugs) did not produced the significant effect on the 8th day, DDE (400 mg/kg) and ASE (200 mg/kg) showed significant effect (**P<0.01) where as ASE (400 mg/kg), C_2 (100 mg/kg each drugs), Piracetam (200 mg/kg) and BM (Bacopa Moneri) (100 mg/kg) increased the IR and decreased the TL significantly (***P<0.001) on 8th day of experiment compared to the amnesic control as the results are shown in table 7. ASE found to be more effective than DDE whereas Combination C_2 produced better effect comparative to the standard drugs.

In this test, DDE (200 mg/kg) and Combination C_1 (50 mg/kg each drug) did not produce a significant effect on the 8th day**,** DDE (400 mg/kg), ASE (200 mg/kg) showed a significant effect (**P<0.01) where as ASE (400 mg/kg), C_2 (100 mg/kg each drug), Piracetam (200 mg/kg) and BM (Bacopa Moneri) (100 mg/kg) significantly (***P<0.001) on the 8th day of experiment compared to the amnesic control, as the results are shown in table 9.

Table 9. The effects of DDE, ASE, C_1, C_2, Piracetam and Bacopa moneri (BM) on learning performance by elevated plus maze test

S. No.	Groups (mg/kg p.o.)	Transverse latency (Sec)		Inflexion ratio
		On 7th day	On 8th day	
1	Control (vehicle 5 ml/kg)	144.50±4.4	62.33±4.93	1.32
2	Amnesic Control (AC) Scopolamine (SC)1	152.75±4.92^a	88.00±12.57^a	0.72^a
3	SC 1+DDE 200	128.11±2.22	66.45±9.44^{ns}	0.93
4	S 1+DDE 400	121.09±6.10	43.03±11.01**	1.81
5	SC 1+ASE 200	129±1.97	47.67±2.10**	1.74
6	SC 1+ASE 400	138.33±3.9	44.54±1.64***	2.13
7	SC 1+Combination C_1 50	131.27±9.12	74.92±12.14^{ns}	0.77
8	SC 1+Combination C_2 100	112.76±214	39.19±9.24***	3.30
9	SC +Piretam 200	111.21±3.21	27.31±0.92***	3.11
10	SC 1+BM 100	131.45±5.32	34.23±3.21***	2.85

Each value represents the Mean±SEM, (n=6), ns= non significant, ^{a}p<0.05 compared to the control, *p<0.05, **p<0.01, ***p<0.001, (ANOVA followed by Dunnett's test against with the amnesic control).

In this test, DDE (200 and 400 mg/kg) and Combination C_1 (50 mg/kg) did not produce a significant effect on the 8th day**,** ASE (200 mg/kg), showed a significant effect (*P<0.01), ASE (400 mg/kg), and Combination of the drugs (100 mg/kg), Piracetam (200 mg/kg), and BM **(**Bacopa Moneri**)** (100 mg/kg) increased the IR and decreased the EL significantly (***P<0.001) on the 8th day of the experiment compared to the amnesic control (AC) as the results are shown in table 10.

Table 10. The effects of DDE, ASE, C_1, C_2, Piracetam and Bacopa moneri (BM) on learning performance by moris water maze test

S.No.	Groups (mg/kg, p.o.)	Escape latency (Sec)		Inflexion ratio
		On 7th day	On 8th day	
1	Control (vehicle 5 ml/kg)	167±10.12	76.14±9.03	1.19
2	Amnesic Control (AC) Scopolamine (SC)1	257±6.12[a]	149.47±9.03[a]	0.872[a]
3	SC 1+DDE 200	166.10±9.09	129.21±11.21 [ns]	1.10
4	S 1+DDE 400	121.59±17.01	112.12±7.13 [ns]	1.88
5	SC 1+ASE 200	200.13±13.12	69.12±9.06**	1.89
6	SC 1+ASE 400	127.5±11.78	42.13±7.02**	2.02
7	SC 1+Combination C_1 50	163.01±8.19	136.01±10.29 [ns]	1.29
8	SC 1+Combination C_2 100	131.02±2.07	29.09±4.76***	3.24
9	SC 1+Piretam 200	93.81±4.73	20.15±09***	3.65
10	SC 1+BM 100	123.12±3.41	37.22±7.19**	2.54

Each value represents the Mean±SEM, (n=6), ns= non significant, [a]p<0.05 compared to the control, *p<0.05, **p<0.01, ***p<0.001, (ANOVA followed by Dunnett's test against with the amnesic control)

In this test, DDE (200 mg/kg), combination C_1 (50 mg/kg each drug) did not produce a significant effect on the 7th day, DDE (400 mg/kg), ASE (200 mg/kg) showed a significant effect (*P<0.05) whereas ASE (400 mg/kg), combination C_2 Piracetam (100 mg/kg) and BM (100 mg/kg) decreased AchE level significantly (***P<0.001) on the 7th day of the experiment compared to the amnesic control, as the results are shown in table 11.

Table 11. The effects of DDE, ASE, C_1, C_2, Piracetam and Bacopa moneri (BM) on AchE level of rat brain.

S.No.	Groups (mg/kg, p.o)	AchE (mol/min/g)
1	Control (vehicle 5 ml/kg)	7.54±0.321
2	Amnesic Control (AC) Scopolamine (SPA)1	22.13±0.421[a]
3	SC 1+DDE 200	18.23±0.021
4	SC 1+DDE 400	14.23±0.021*
5	SC 1+ASE 200	15.68±0.011*
6	SC 1+ASE 400	10.68±0.011 **
7	SC1+Combination C_1 50	18.41±0.05[ns]
8	SC 1+Combination C_2 100	9.31±0.04**
9	SC 1+Piretam 200	8.54±0.02***
10	SC 1+BM 100	12.41±0.035**

Each value represents the Mean ±S EM, (n=6), ns= non-significant, [a]p<0.05 compared to the control, *p<0.05, **p<0.01, ***p<0.001, (ANOVA followed by Dunnett's against with the amnesic control)

DISCUSSION:

In the recent work, it was observed that DDE and ASE (200 and 400 mg/kg) possessed the anti-stress activity, antioxidant and nootropic activity.

In swimming endurance test, it was observed that treatment with DDE and ASE (200 and 400 mg/kg), Combination C_1 and C_2 (each drug 50 and 100 mg/kg) and Withania somnifera (100mg/kg) for 7 days significantly increased in the swimming endurance time and duration of stay on rota rod. The results of the recent work revealed that ASE, DDE and combination of both the drugs increased swimming endurance and post swimming muscle coordination performance (physical stress) in the animals have significant anti-stress

activity. It may be because of bringing back to normal blood plasma level of catecholamine and monoaminoxidase [29].

In the immobilization stress trial, the all biological parameters like cholesterol, glucose, TG, BUN and haematological parameters like RBC and WBC count were significantly decreased by the pre-treatment of DDE and ASE (200 and 400 mg/kg), Combination C_1 and C_2 (each drug 50 and 100 mg/kg) and Withania somnifera (100mg/kg) when compared to the stress control group. The possible mechanism of action of the tests drugs to decreased the stress related increased level of cholesterol is may be due to decreased liberation of catecholamines, corticosteroids and adrenaline which are responsible for elevation of serum cholesterol level in response to stress stimuli [30]. In the present study immobilization stress increased the triglyceride levels whereas ASE, DDE and combination of both the drugs decreased the stress related increased the triglyceride (TG) level. The test drugs ASE, DDE and combination of both the drugs decreased the BUN levels as compared to stress control in this test. Stress increased the adrenocortical activity which leads to increase the metabolism of protein results in excess level of BUN [31]. Thus, theses drugs demonstrating a decreased catabolism of protein under stressful situation**.** Immobilization stress generally increases total WBC and RBC count due to increased oxygen demand, to compensate these extra demands, RBC and WBC count increases [23]. Pre-treatment with ASE and DDE reduced the stress-induced elevated levels of haematological parameters like RBC and WBC in immobilization stress. In this test, weight of liver and adrenal glands were significantly increased. Stress stimulates the adreno-medullary response to liberate adrenaline which in turn triggers β2 receptors on the pituitary gland. It leads to bigger discharge of adrenocorticotropic hormone (ACTH) that can excite the adrenal gland resulting in additional release of adrenaline and elevate the weight of adrenal gland to a greater extent. The adrenal hypertrophy takes place in response to the secretion of ACTH from the pituitary for increased corticosterone from cortical cells to combat stress [32]. Spleen becomes small in size during stress and releases more amount of blood (RBC) into circulation; hence its weight decreases [31]. Pre-treatment with DDE and ASE, combination of the drugs and standard drug WS prevented the stress-induced increase in weight of liver, adrenal glands and stress-induced decrease in testis and spleen indicating the defensive work against stress.

Low doses of DDE, ASE and combination of the drugs significantly whereas high doses more significantly increased the anoxia time on last day compared to the control group. This effect may be because of elevating succinat dehydrogenase (SDH) level in the rat brain during stress. In the cellular system of an organism, SDH is responsible for consumption and preservation of energy which helps adaptive processes during stress [33]. In other way, the anti-hypoxic effect of the test drugs may also be due to improved or raised cerebral resistance to hypoxia and reduced cerebral utilization of oxygen in acute hypoxia. In hypoxic condition, the mitochondria of heart and brain cells are seriously damaged. Brain neurotransmitters, i.e. norepinephrine, dopamine, serotonin and acetylcholine level are significantly decreased. Our results demonstrated that the test drugs exhibited significant and dose dependant antistress activity as indicated by increase in duration of anoxia stress tolerance time [19]. The results of recent work also indicated that synergistic effect of high dose of both the drugs (Combination C_2 100 mg/kg) showed better anti-stress effect and ASE was found to be more effective compare to the DDE.

Antioxidant activity of different extracts of DDE and ASE were determined by DPPH and Nitric oxide methods following in vitro methods. These methods are most popular in vitro assays for determination of antioxidant activity. DPPH scavenging assay is an important assay to determine the antioxidant activity of the plant extracts in in-vitro model. DPPH is free radical which is reacting rapidly with the antioxidant compounds. The anti-oxidative compounds can donate a hydrogen atom to DPPH and change the color. The intensity of color is measured calorimetrically. The increasing intensity of color is directly proportional to the inhibition of DPPH [34]. The present study showed the increasing concentration of the test drugs extracts (ASE and DDE) inhibit the activity of DPPH. The maximum inhibition was noticed at 1500 μg/ml.

The reducing power is related to electron transfer ability of the plant extract. In this assay is used to measure the transferring capacity of Fe3+ to Fe2+ [35]. The results showed that the test drug extracts possessed antioxidant activity in a concentration dependent manner. Based on the results, it was found that ASE and DDE have a ability of transferring the Fe3+ in to Fe2+ and it minimize the oxidative damage in the tissues.

Nitric Oxide is a free radical which is formed from sodium nitroprusside and it reacts with oxygen to form nitrite. The antioxidant activity was measured by the inhibition of the nitrite formation, this was done by the

plant extracts which directly reacts with oxygen, nitric oxide and other nitrogen compounds [36]. The present study proves that the increasing concentration of the extract have a maximum inhibitory activity against the nitric oxide.

Hydrogen peroxide is an important reactive oxygen species because it may be toxic if it is converted into hydroxyl radicals in the cells [37]. Antioxidant compounds donate electrons to H2O2, neutralising it into a water molecule [34. The present study proves the inhibition of hydroxyl radicals by H_2O_2 in a concentration dependent manner.

Antioxidant power is indirectly proportional to the IC_{50} value. Lower the IC_{50} value greater the antioxidant activity. In these tests, standard drug ascorbic acid showed more antioxidant activity compare to the ASE and DDE. ASE was found to be better than the DDE. ASE produced comparable effect to the standard ascorbic acid.

Alziemer disease (AD) is a genetically heterogeneous neurodegenerative disorder, which is slow in onset but relentless in progress. It is characterized by aphasia, apraxia and agnosia with loss of memory as the main symptom [38]. Among the various approaches attempted to increase cholinergic activity, the inhibition of Acetylcholiesterase (AChE) is the most successful one [39]. Cholinesterase Inhibitors (ChEI) are the only class of compounds consistently proven to be efficacious in treating the cognitive and functional symptoms in patients with neurodegenerative disorders such as AD, Parkinson's disease, senile dementia, ataxia and myasthenia gravis etc [40]**.** In addition, new findings showed that both AChE and Butyrylcholinesterase (BChE) are involved in the breakdown of acetylcholine in the brain and, thus, dual inhibition of these enzymes may prove efficient in treating dementia [41].

Learning has been defined as the process acquiring the knowledge while memory is the retention of the acquired knowledge that can be retrieved [42]**.** The elevated plus maze test (EPMT) is used to measure the anxiety state in animals, however, transfer latency i.e. the time elapsed between the movement of the animal from an open to an enclosed arm was markedly shortened if the animal had previously experienced entering open and closed arms, and this shortened transfer latency has been shown to be related with memory processes. Recent studies of several nootropics and amnestic agents on EPMT made this model a widely accepted paradigm to study learning and memory processes in rodents [43]**.**

In elevated plus maze and Morri's water maze, acquisition (learning) can be considered as transfer latency on 7^{th} day trials and the retention/consolidation (memory) is examined 24 h later that is on 8^{th} day. On 8^{th} day, Scopolamine is a anti-cholinergic drug that decreased the level of acetylcholine in brain and impaired the memory. Scopolamine treated group decreased inflexion ratio (IR) as compared to normal control group indicated the induction of amnesia. The test drugs DDE and ASE (200 and 400 mg/kg), combination of both the drugs C_1 and C_2 increased IR (decreased the TL in EPMT and EL in MWMT) in dose dependent manner which is an indication of increasing acetylcholine level might be inhibiting/decreasing the AChE enzyme level in brain and test drugs antagonized the Scopolamine effect showing the memory enhancer effect [44]. Further study of estimation of Acetylcholine esterase enzyme (AChE) in rat brain indicated that the test drugs DDE (200 and 400 mg/kg), ASE (200 and 400 mg/kg), combination of both the drugs decreased AChE enzyme level in dose dependant manner. Among all the groups, Combination C_2 (100 mg/kg) produced the better effect, ASE was found to be better than the DDE. Our results suggest that the anti-amnesic effect of ASE and DDE in present study would be due to its antioxidant action.

CONCLUSION

The study showed that hydroalcohlic extracts of both the drugs (DDE and ASE 200- 400 mg/ kg) and combination C_2 (100 mg/ kg) possessed anti-stress, antioxidant and nootropic activities. Further research is required to be done to evaluate the real mechanism of action of both the drugs and responsible phytochemical presents in the drugs. The synergistic effect of both the drugs showed better effects. In this work, results of present work also indicated that ASE was found to be more effective compare to the DDE.

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