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Neuroprotective Effects of *Asparagus racemosus* Willd and *Withania Somnifer*a Dunal: An *In Vitro* And *In Vivo* Study

Durgesh Sharma
Department of Zoology, M.P. Government P.G. College, Chittorgarh, Rajasthan, India

ABSTRACT

Aims of the study was to investigate and compare the protective effects of two plants *Asparagus racemosus* Willd (AR) and *Withania somnifera* Dunal (WS) in *in vitro* and *in vivo* models of neurodegeneration. Further, it also tested that the protective action was due to antioxidant activity of both the plants. *In vitro* study was carried out in hippocampal neuron cells in primary culture and *in vivo* in young adult Wistar rats. Root extracts of *A.racemosus* and *W.somnifera* were obtained from a authorized supplier. To asses neuroprotective action of the plants, cell viability tests (AO/EB staining, MTT assay, LDH leakage assay), extracellular Free Ca^{2+}, ROS determination, GSH-GSSG ratio and lipid peroxidation were studied in *in vitro*. In *in vivo* study, ROS determination, Free Ca^{2+}, GSH-GSSG ratio, GPx level and lipid peroxidation were determined in hippocampal tissue. In addition dose response and exposure time response was also studied in *in vitro*. Results suggested that in culture, significant decrease in dead cells was observed after treatment with WS as compared to AR+WS. MTT assay also confirmed these observations. A significant decrease in Free Ca^{2+}, ROS and lipid peroxidation and increase in GSH-GSSG ratio was observed. In hippocampal tissue as well , a significantly decrease in ROS level, Free Ca^{2+}, GPx level and lipid peroxidation but increase in GSH-GSSG ratio was observed. Study thus demonstrates that AR and WS both have potent neuroprotective action and that this effect is due to their antioxidant properties.

Keywords: *A.racemosus,* Antioxidants, Hippocampal cell culture, Neuroprotection, *W.somnifera,*

I. INTRODUCTION

The brain is relatively more susceptible to free radical damage than other tissues (Halliwel, 2006) . In brain, hippocampal neurons are particularly more vulnerable to detrimental effects of oxidative stress. Cell loss in hippocampus during normal aging and during neurodegenerative disorders like Alzheimer's, Parkinson's, has been linked to deficit in endogenous antioxidant machinery (Beal, 2004; Liu et al., 2007). Thus efforts are on to identify natural molecules which have strong antioxidant and free radical scavenging properties to develop alternative therapy (Anaconda and reddy, 2005; Dos-Santos et al., 2006). Several herbal preparations have been tested in culture (Jing et al., 2008; Limpeanchob et al., 2008; Tateno et al., 2009) or animal models of the disease as well as in clinical trials (Basianto et al., 2000; Bhatnagar et al., 2000; Shukla et al., 2000; Jain et al., 2001; Sankar et al., 2009), for their free radical scavenging and neuroprotective action. Other treatments for neurodegenerative disorders includes use of acetylcholinesterase inhibitors, anti-inflammatory agents and β-amyloid based immunotherapy (Weggenn et al., 2007; Soloman, 2007) to maintain cognitive functions of patients.

Asparagus racemosus (Shatawari, AR) is a traditional medicinal plant used in India not only as antiepileptic drug but also to enhance intelligence and promote learning and memory (Sharma, 1993). The herb also known to improve health by increasing immunity, vitality and protection against stress (Puri, 2003). Active constituents of *A. racemosus* like flavonoids, oligosaccharides, amino acids, sulfer containing acids

and steroidal saponins are known to possess antioxidant properties (Saxena and chourasia, 2001; Wiboonpun et al., 2004; Parihar and Hemnani 2004). *Withania somnifera* (Ashwagandha, WS) categorized as rasayana is another plant used to promote health, longevity and arrest aging process (Weiner and weiner, 1994). Plant has been used as an antioxidant, adaptogen, anti-inflammatory agent, for treating senile dementia and Parkinson's disease (Gupta and Rana, 2007). Clinical trials and animal research support the use of *W. somnifera* for anxiety, cognitive and neurological disorders and in Alzheimer's and Parkinson's disease (Bhattacharya et al., 2000; Bhattacharya and Murogannandam, 2003; Ahmed et al., 2005). The active constituents in WS include alkaloids, steroidal compounds, saponins, sitoindosides VII-X and withaferin A (Gupta and Rana, 2007; Bhattacharya, 1997) tested for antioxidant activity. Cognition enhancing and memory improving effects of W*. somnifera* extract were also reported (Schelibs et al., 1997).

This study is therefore focused to investigate the effects of treatment of two plants *Asparagus racemosus* Willd (AR) and *Withania somnifera* Dunal (WS) in *in vitro* and *in vivo* models of neurodegeneration. It was hypothesized that mode of neuroprotective action of these drugs involve enhancement of antioxidant potential besides directly quenching the free radicals.

II. METHODS AND MATERIAL

Preparation of extract

A. racemosus (AR) and *W. somnifera* (WS) crude extracts were purchased from R and D center, Indian Herbs Research Supply Co. Ltd., Saharanpur, UP, India.

In vitro studies

Preparation of primary hippocampal cell culture (Sunanda et al., 1998) *and treatments.*

New born (0 day) pups of Wistar rats were used. Hippocampi of two pups were dissected out under stereo zoom microscope and pooled in 2 ml of the culture medium in a sterile petri dish. Tissue was then mechanically triturated through 18 gauge needle attached to a 5 ml syringe. Cells were collected by centrifugation at 1000 rpm for 5 min and resuspended in DMEM, supplemented with 10% FBS. Cells were suspended in media and single cell suspension was seeded in 96 well plates coated with 0.01% poly-L-lysine, at a density of $5x10^4$ cells/well. Ampicillin and Streptomycin were added to the medium. Cells were cultured at 37^0C in presence of 5% CO_2 , and 100% humidity for 7 days before using them for further experimentation. The medium was replaced every alternate day. After 7 days cells were treated with glutamate (Glu) to induce oxidative stress. For treatment, wells were divided into seven groups: I. Control II. Glutamate, III. Glu+AR, IV. Glu +WS, V. Glu +AR+WS, VI. AR, and VII. WS groups. 100 µm glutamate was added to the medium for 10 minutes at room temperature in HBSS. AR and WS extracts (10µg/ml) were added to medium for one hr before glutamate treatment. After glutamate treatment, culture was washed twice with HBSS and replaced with fresh culture media containing plant extracts. Culture dishes were returned to the incubator for 24 hrs prior to various analyses. Control group cells were treated with PBS alone or AR and WS extract only.

Dose response assay

For selection of an optimum dose concentration and duration of incubation period , a dose response assay was carried out for *in vitro* study. Cell survival assay in primary hippocampal culture was carried out against glutamate treatment in the presence of various concentrations (1-30 µg/ml.) of plant extracts and for varying exposure duration (2-72 hrs).

Cell viability assay

Cell viability was assessed by using AO/ EB (Acridine orange (AO)/ Ethidium bromide (EB)

staining (Spector et al., 1999) and by MTT assay (Hansen et al., 1989. In AO/EB assay viable and dead cells were counted using haematocytometer. Mean dead/viable cell count was determined in control and experimental groups. Percent cell viability was determined taking control as 100%. In MTT assay % MTT reduction was observed in each well after different treatments.

ROS estimation

ROS level was measured by the method of Myhre et al., (2003). Hipocampal cells were incubated with DCFDHA (5µg/ml) for 40 minutes at 37 ^{0}C in dark. Fluorescence intensity was quantitatively estimated spectrofluorimetrically with excitation at 485 nm and emission at 530 nm, in control and all the treatment groups. Results obtained were calculated in terms of arbitrary fluorescent units (AFU) per mg of protein and expressed in % taking control as 100%.

Free intracellular Ca^{2+}

Intracellular Ca^{2+} was measured using Ca^{2+} sensitive dye Fluoro-3 AM (Mattson e t al., 1993). Non fluorescent Fluoro-3AM is converted into Fluoro-3 by intracellular esterase, which then binds with Ca^{2+} and become fluorescent, which can be measured fluorometrically. Cells were incubated with 5µM Fluoro-3 AM for 30 minutes in HBSS. Cells suspension was then prepared by trypsinization. Fluorescence was measured in all groups at 506 nm excitation and 526 nm emission and Ca^{2+} concentration was expressed as AFU.

LDH leakage

LDH leakage corresponds to cytotoxicity and membrane damage. Enzyme activity was thus estimated (U/mg/ protein) and expressed as a percentage, taking control as 100%, following method of Koh and Choi (1987).

GSH and GSSG estimation

The antioxidant status was measured in terms of GSH and oxidized glutathione (GSSG) as described by Hissin and Hilf (1976). The amount of GSH and GSSG were calculated from the standard curve and expressed in µM /mg protein. Values were expressed in percentage, taking control as 100%.

Lipid peroxidation

MDA (Malondialdehyde) the end product of lipid peroxidation was measured spectrophotometrically (Utley et al., 1976). MDA formed was estimated using molar extinction coefficient of MDA-TBA complex (1.56 x 105 cm^2 mMol-1) and the values obtained were expressed in % with respect to control.

In vivo studies

Animals : Male Wistar rats (BW 150±2) g were used for the study. Rats were kept in controlled conditions of temperature (22±2^{0}C), humidity and light-dark cycle (12 hrs -12 hrs). Animals were divided into control (n=6) and six experimental groups (n=6) and each animal was assigned to these groups as per randomized number table.

Treatments

Experimental groups were – Control, Stress, Stress+AR, Stress+WS, Stress+ AR+WS, AR and WS groups. For stress treatment, animals were subjected to restraint stress. Animals were kept in tightly fitted ventilated plastic boxes for 4 hrs daily up to 15 days. For dose preparation and treatment, extracts were dissolved in 0.5% carboxymethyl cellulose (CMC) in distilled water. All experimental animals were administered with plant extracts (40 mg/Kg BW) one hr before stress treatment. Dose of WS and AR extracts for *in vivo* study was decided empirically on the basis of previous studies (Bhatnagar et al, 2009; Bhattacharya et al., 2001). AR and WS extract was administered orally using feeding tube. Single dose was given every day and continued up to 30 days from the day of stress treatment. Control animals were given same dose of vehicle.

Tissue preparation

Animals were sacrificed under pentobarbital anesthesia. Brain was quickly dissected out and hippocampi were removed on ice cold plate and minced. 10% homogenate was then prepared in 0.1M phosphate buffer. Crude homogenate was used for MDA, GSH , GSSG assay. For ROS and LDH determination homogenate was centrifuged at 10,000g for 10 minutes and supernatant was taken for estimation. Total protein content was estimated by method of Lowry et al., (1951).

ROS estimation

Le Bel et al., (1999) method was used. Briefly, homogenate was incubated with DCFHDA for 15 minutes at 37^0C in dark and fluorescence was measured at 488 nm excitation and 525 nm emission. Intensity was expressed as fluorescent units/mg protein.

Cytosolic free Ca^{2+}

For determining the cytosolic free Ca^{2+} concentration the homogenate was centrifuged and supernatant was incubated with 5μM Fluoro-3 AM for 30 minutes in dark according to the method of Mattson et al., (1993) . Fluorescence was measured spectrophotometrically at 560 nm excitation and 526nm emission. Free Ca^{2+} was expressed as AFU.

GSH and GSSG assay

Brain GSH and GSSG estimation was performed as described earlier in vitro studies. Briefly, homogenate was mixed with equal volume of metaphosphoric acid and then centrifuged at 10 000g. Supernatant obtained was used. Values were expressed as μM/mg protein.

GPx assay

GPx (Glutathione peroxidase) enzyme catalyses the reduction of various organic hydroperoxides as well as H_2O_2 with glutathione as hydrogen donor. 10% tissue homogenate was prepared in 0.1M PBS (pH7.4), and centrifuged. Supernatant was used for measurement and reading was taken at 412 nm and represented as moles of GSH utilized /mg protein/min.

Lipid peroxidation determination

MDA was measured by the method of Buege et al., (1978). MDA react with TBA to form pink chromogen which was measured by taking absorbance at 532 nm spectrophotometrically.

III.RESULTS AND DISCUSSION

Results

Evaluation of the effects of AR and WS extract on hippocampal cells in culture-

At the concentration of 10 μg/ml both plant extracts showed maximum neuroprotection in the primary neuronal culture exposed to glutamate. Up to 4 μg/ml concentration no significant change in cell survival was observed. A linear increase in the cell survival was observed between the concentration range of 6-10 μg/ml, which was stable up to 15 μg/ml and a gradual decrease was observed after this concentration (Fig.1A). Therefore, 10 μg/ml concentration was selected to optimize incubation period. Glutamate treated cells were incubated with WS and AR extracts for 2hrs, 6hrs, 12 hrs, 24hrs, 48 hrs and 72 hrs (Fig. 2B). Results showed that up to 6 hrs of incubation no significant change in the cell survival was observed, after 12hrs of incubation cell survival was increased gradually up to 24 hrs and after that it became stable and further no significant increase was observed till 72 hrs. On the basis of assay we selected 10 μg/ml concentration and 24 hrs incubation time for further experimentation.

Cell viability was confirmed by counting AO/EB stained cells and by measuring MTT reduction in all experimental and control groups. A significant increase in number of dead cells was observed in glutamate treated group (Fig.2B), as compared to control group (Fig.2A; taken as 100% survival). When cells were treated with AR, WS and

AR+WS before glutamate treatment, % of viable cells was at par with control group (Fig.3A). MTT reduction was found increased after glutamate treatment nearly 45% when compared to control group (P<0.001). In AR , WS and AR+WS treated Glu group, a significant decrease in MTT reduction was observed . In AR+WS treatment Glu groups, decrease in MTT reduction was more significant (P<0.001), when compared with AR, WS and control groups (Fig.3B).

In vitro antioxidant activities

Antioxidant activity of AR and WS was determined in primary culture of hippocampal cells, using ROS assay, free Ca^{2+} , LDH leakage, GSH and GSSG assays, and lipid peroxidation inhibition.

Results showed, reduced ROS as evidenced by significant reduction in fluorescence in Glu+ AR and Glu+WS group compared to only Glu treated group (Fig. 3C). There was no significant difference between control and Glu+ AR +WS group (p>0.05).

As a result of glutamate induced excitotoxicity, more than two fold increase in free Ca^{2+} concentration was observed in glutamate treated cultured neurons when compared with control (Fig. 2D). This increase was inhibited significantly (p<0.01) in Glu+ WS group compared to only Glu treated group. No significant effect of AR was observed on Ca^{2+} concentration (p>0.05; Figs.2C-2F).

LDH leakage, a marker of membrane damage, showed about 150% increase in Glu treated group when compared with control. A significant decrease (p<0.01) in LDH leakage was observed in Glu+ AR+WS <Glu+ AR < Glu+ WS treated groups when compared with only Glu treated group (Fig. 3E).

GSH level declined significantly (p<0.01) after treatment with Glu as compared to control group (Fig. 3F). A s compred to Glu group, Glu+Ar+WS group showed more significant increase then Glu+AR and Glu +WS(p<0.01).

GSSG level in glutamate group increased significantly (p<0.001) when compared with control group. Increase of GSSG level (Fig.3G) in herbal extract treated groups was significantly less than only glutamate group. However, a increase in Glu+WS and Glu+ AR was more significant than Glu+AR+WS (p<0.001).

MDA level increased more than two fold in Glu treated group as compared to control group (p<0.001). Presence of AR or WS extract prevented increased lipid peroxidation after glutamate treatment. Significant decrease in lipid peroxidation was observed after Glu+AR, Glu+WS as well as Glu+AR+WS treatment, when compared with glutamate group (p<0.01; Fig. 3H)

Evaluation of in vivo antioxidant effects of AR and WS extracts

GPx activity was measured in terms of utilization of GSH (substrate) per minute in tissue homogenate. Almost three fold decrease in activity of enzyme was observed in stress group. A significant increase in activity was observed in Str+ WS, Str+AR and Str+WS+AR treated groups (Fig. 4A).

GSH level was significantly low in stress group as compared to control group (p<0.001). Increase in GSH level was shown in Str+WS and Str+AR in comparison to Str+WS+AR treated group when compared with only stress treated group, however this increase was not significant in AR treated group (Fig. 4B).

GSSG level in stress group was significantly high as compared to control group (p<0.01). This increase in GSSG level was significantly reversed in Str+AR, Str+ WS and Str+ WS+AR treated groups (Fig. 4C)

Similar to enzyme GPx , Str+WS, Str+AR showed significant (p<0.01) decrease in lipid peroxidation, but no significant (p>0.05) difference was observed in str+WS+AR treated group (Fig. 4D).

Significant increase in ROS level was observed in stress group as compared to control group (p<0.01). Treatment with AR, WS to stress animals showed more significant decrease in ROS then WS+AR, when compared with stress group (Fig. 4E).

Stress results in significant increase in free Ca^{2+} concentration ($p<0.01$) when compared with control. This increase was significantly ($p<0.01$) reversed in groups treated with WS extract (Str+WS), whereas no effect of AR extract was observed in group Str+AR (Fig. 4F). Stress group showed significant increase in lipid peroxidan when compared to control ($p<0.01$).

Discussion

Results of the present study demonstrate neuroprotective effects of AR and WS studied in *in vitro* and *in viv*o models of neurodegeneration. Study also demonstrates that combined treatment of both plant extract showed synergistic effects. These protective effects can be attributed to their potent antioxidant activity. Earlier, studies on AR (Anaconda and Reddy, 2005; Parihar and Hemnani, 2004; Ahmed et al., 2005), against KA induced hippocampal and striatal neuronal damage model have also shown decline in oxidative stress after treatments with AR and WS extracts, indicating that plant extracts have primary role as an antioxidant. The results of this study also confirm that there is a significant increase in oxidative stress in culture hippocampal cells after glutamate treatment and in rat brain after exposure to restrain stress, which was evident by increase in ROS level, MDA, LDH leakage and decline in GSH- GSSG ratio, both in culture cells and hippocampal tissue. MDA is a end product of lipid peroxidation and its high value represent significant denaturation of poly-unsaturated fatty acids etc., in cell membranes which ultimately result in apoptotic cell death. LDH leakage is also known to correspond to cytotoxicity and membrane damage. GSH is an important antioxidant that delimits oxidative damage caused by ROS. It acts as scavenger as well as substrate for GPx mediated destruction of hydrogen peroxide(Meister, 1983). Thus as the brain tissue is deficient in antioxidant enzymes SOD and CAT (Cardozo- pelaez et al., 2000), GSH content must be depleted below certain threshold level in order for increase in lipid peroxidation and resultant cell death (Tirmenstein et al., 2000). Parihar and Hemnani (2004) and Weiner and weiner (1994) have discussed that AR and WS treatment concomitantly increase GPx level and GSH with decline in lipid peroxidation both in KA induced neurotoxicity and in normal brain. Thus protective effects of AR and WS were attributed to improved antioxidant status of cells. Our observation of significant increase in GSH- GSSG ratio after AR , WS and AR+WS treatment in both normal cell culture and hippocampal tissue as well as in glutamate treated culture and in hippocampal tissue are in conjunction with these reports and clearly demonstrate the antioxidative effects of the two herbs. In this study, we also observed changes of intracellular Ca^{2+} both *in vitro* and *in vivo* studies. A significant increase in $Ca^{2+}$was observed after glutamate treatment in culture and in hippocampal tissue after restrain stress. Glutamate exerts its neurotoxic effects through both activation of NMDA receptor and nonNMDA glutamatergic receptor activation (Olney , 1969), resulting in to influx of Ca^{2+} in to the cells Michaels and Rothman, 1990). Similarly, restrain stress disturb glucorticoid homeostasis and thus cause activation of glucorticoid receptors which render hippocampal neurons in brain to oxidative stress via glutamate receptor activation (Lowy et al., 1993). Activation of glutamate receptor trigger excessive entry of Ca^{2+} initiating series of cytoplasmic and nuclear process to promote neuronal death. Rise in Ca^{2+} also activates nNOS which in turn react with peroxide to produce peroxynitrite a strong oxidant (Calabrese et al., 2007). Treatment with AR and WS showed that WS alone showed significant decline in Ca^{2+}. This indicate that WS extract could be more effective in reducing Ca^{2+} induced oxidative readicals as compared to AR. In a earlier study from this laboratory, we have shown that WS extract effectively protects against stress induced glutamate toxicity and inhibits nNOS production and NO production (Bhatnagar et al., 2009). This study, clearly suggest Ca^{2+}buffering action

of WS, suggesting strong protective effects. We also gathered cell counting data using Acridine orange (AO)/ ethidium bromide (EB) double staining in culture cells. AO /EB staining demonstrated significantly decreased AO stained cells after treatment with AR and WS extract as compared to glutamate treated groups. In addition, cell viability assay was carried out with the aim to evaluate the effects of both extracts on cell survival. MTT assays showed significant increase in MTT reduction after AR and WS treatment.

AR and WS have been used for thousand of years in Ayurveda as Rasayana drugs and in Indian traditional medicinal system for treatment of various nervous ailments and also to prevent aging, enhance cognitive functions and learning and memory (Puri, 2003). Several reports (Sankar et al., 2009; Kamat et al., 2000; Gupta et al., 2003), have shown antioxidant activities of these plants including reports from this laboratory. Thus these plant could be useful in preventing neuronal degeneration in brain and could provide better agents for treatment of neurodegeneration. Protective effects of these plants could be explained on basis of there strong antioxidant activity as both plants are rich in polyphenols which have ability to bind to specific groups or proteins on cell membrane and thus suppress the cascade of enzyme reactions leading to cell death.

IV.CONCLUSION

In conclusion, study thus demonstrates that AR and WS both have potent antioxidant properties . This activity could be reason for their neuroprotective action. Taken all the evidences together it appears that WS and AR + WS in combination exert more protective effects then AR alone. Therefore, AR and WS could be useful for developing alternative therapy for neurodegenerative disorders.

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Legends to figures:

Fig.1- A: Effect of various concentrations of WS and AR extracts on cell viability in primary hippocampal culture after exposure to glutamate.

Fig.1- B: Optimization of incubation period for the 10µg.

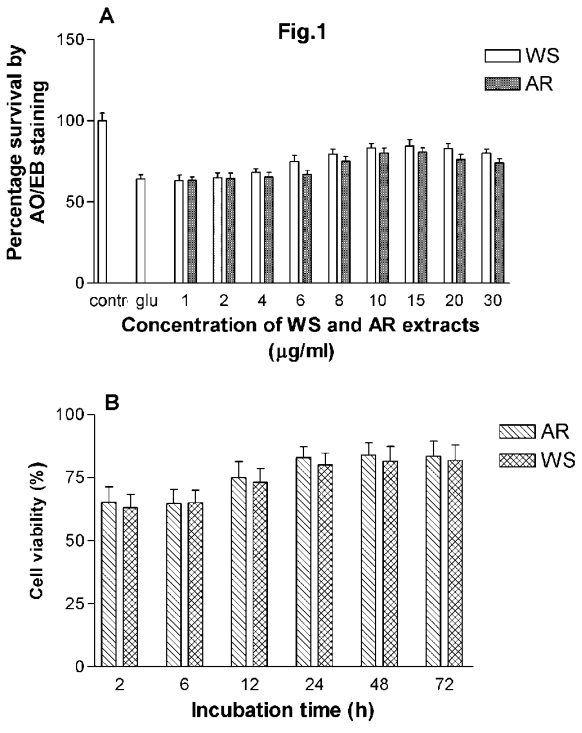
Fig. 2- 2A-2B: Cell viability test in primary hippocampal culture using Ethidium bromide/Acridine orange; 2A- Control group, live neurons stained with acridine orange (green colour); 2B- glutamate treated group, degenerating neurons stained with ethidium bromide (orange colour); 2C-2F: Free cytosolic Ca++ measurement using Fluo 3A, 2C- control, 2D- glutamate, 2E- glutamate+AR, 2F- glutamate+WS.

Fig. 3- Effects of glutamate and extracts of AR and WS in primary hippocampal culture. A-B: Percentage cell survival by AO/EB staining and MTT assay respectively; C: estimation of reactive oxygen species using DCFHDA dye, level of ROS is measured arbitrarily by determining fluorescence units; D: measurement of free cytosolic Ca++ level by using Fluo-3 A, Ca++ concentration is measured arbitrarily by determining fluorescence units. E: LDH leakage; F-G: GSH and GSSG concentration; H: lipid peroxidation measured by calculating MDA concentration; Cont- control, Glu-glutamate, AR- *Asparagus racemosus*, WS- *Withania somnifera.*

All values are expressed as Mean±SD, data comparisons were carried out using one way analysis of variance followed by Bonferroni post test to compare all pairs of groups, * - difference is significant at 5% level of probability, *a – when compared with control, *b – when compared with glutamate treated group.

Fig. 4- Effects of Stress and extracts of AR and WS on rat hippocampus; A: Glutathione peroxidase activity is determined by calculating amount of GSH utilized as µg/min.: B-C: GSH and GSSG concentration; D: lipid peroxidation measured by calculating MDA concentration; E: estimation of reactive oxygen species using DCFHDA dye, level of ROS is measured arbitrarily by determining fluorescence units; F: measurement of free cytosolic Ca++ level by using Fluo-3 A, Ca++ concentration is measured arbitrarily by determining fluorescence units. Cont- control, Str- stress, AR- *Asparagus racemosus*, WS- *Withania somnifera*.

Results are expressed as Mean±SD, conditions as in Fig. 3.



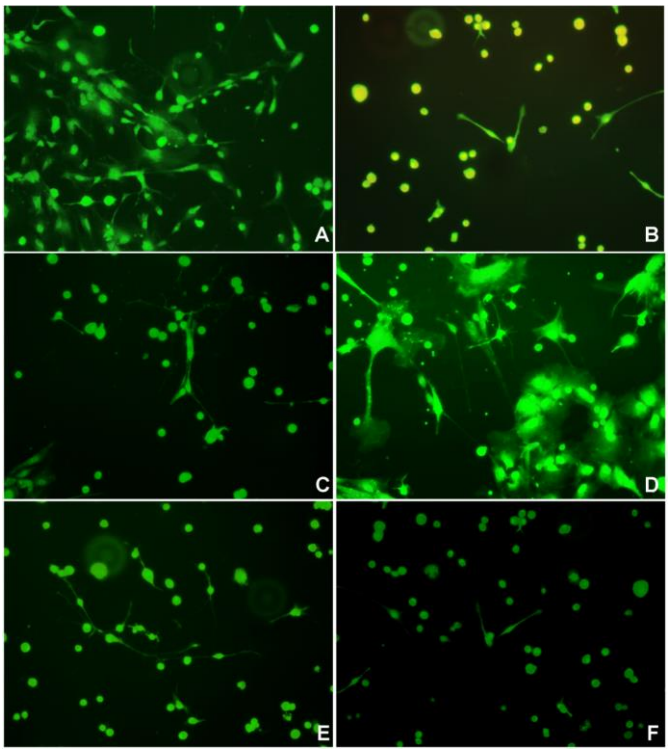

A
B
C
D
E
F

Fig.3

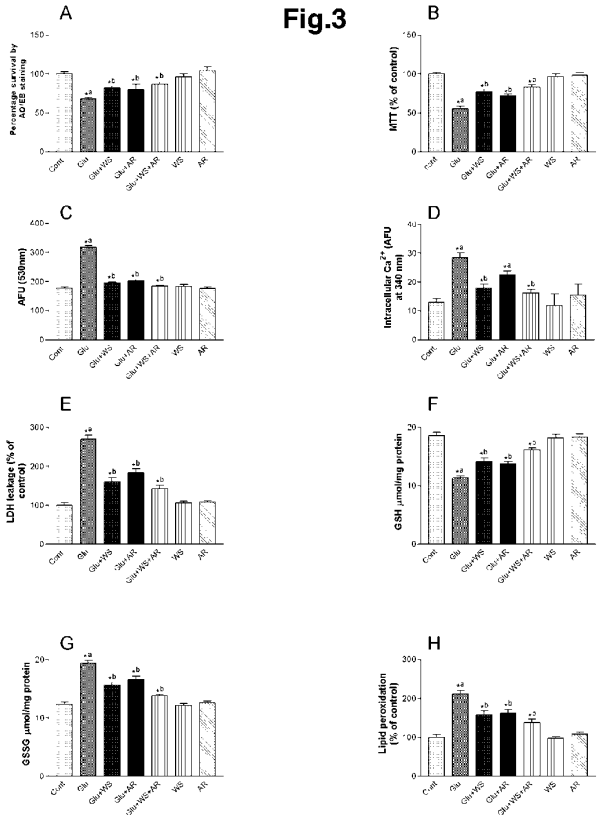
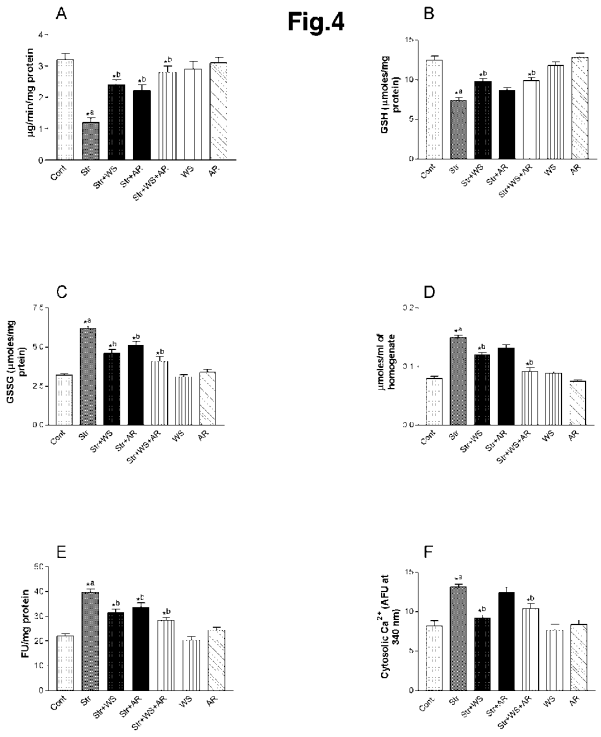

A
Percentage survival by AO/EB staining
B
MTT (% of control)
C
AFU (530nm)
D
Intracellular Ca2+ (AFU at 340 nm)
E
LDH leakage (% of control)
F
GSH µmol/mg protein
G
GSSG µmol/mg protein
H
Lipid peroxidation (% of control)
Cont
Glu
Glu+WS
Glu+AR
Glu+WS+AR
WS
AR

Fig.4


A
µg/min/mg protein
B
GSH (µmoles/mg protein)
C
GSSG (µmoles/mg protein)
D
µmoles/ml of homogenate
E
FU/mg protein
F
Cytosolic Ca2+ (AFU at 340 nm)
Cont
Str
Str+WS
Str+AR
Str+WS+AR
WS
AR