



## EFFICACY OF THE HERB, *WITHANIA SOMNIFERA* L. IN THE PREVENTION OF STRESS-INDUCED ALTERATIONS IN THE OVARY OF RAT

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### ABSTRACT

The present study aimed to investigate the efficacy of the herb ashwagandha in the prevention of stress-induced alterations in the ovary. Exposure of rats to two stressors i.e. restraint (1 h), then after a gap of 4 h to forced swimming exercise (15 minutes) every day for 4 weeks resulted in a significant increase in the adrenocortical activity, indicating the activation of hypothalamo-pituitary-adrenal axis. Concomitantly, there was a decrease in the weight, activities of 3 $\beta$ -hydroxysteroid dehydrogenase and antioxidant enzymes, number of healthy antral follicles, corpora lutea and percentage of healthy granulosa cells of the ovary, whereas the number of atretic follicles, the percentage of apoptotic granulosa cells and malondialdehyde concentration of the ovary showed an increase in the stressed rats compared to those of controls. However, stressed rats pre-treated with the alcoholic extract of ashwagandha (10mg/ kg body weight, oral) did not show these adrenal and ovarian stress responses and did resemble controls. The results for the first time reveal that an extract of ashwagandha is potent enough to inhibit activation of hypothalamo-pituitary-adrenal axis and maintain normal gametogenic and antioxidant activities of the ovary under stressful conditions.

**KEY WORDS:** *Ashwagandha, stress, antioxidant, corticosterone, HPA axis, apoptosis*



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## INTRODUCTION

Stress is a response of an individual to the external or internal stimuli, that affect the homeostasis.<sup>1</sup> In the fast-paced and competitive world, stress has become a major factor affecting several physiological processes of an individual including reproduction. Earlier studies have shown that stress affects reproduction in males as well as females.<sup>2</sup> In females, stress affects several vital processes, viz., folliculogenesis,<sup>3,4</sup> oocyte development,<sup>5</sup> ovulation,<sup>6</sup> steroidogenesis.<sup>7</sup> It is known that stress exerts its effects through the activation of hypothalamo-pituitary-adrenal (HPA) axis.<sup>8</sup> The components of the HPA axis suppress the functioning of hypothalamo-pituitary-gonadal (HPG) axis.<sup>7</sup> Glucocorticoids (GC), being the principle components of the HPA axis, suppress HPG axis on one hand<sup>7</sup> and on the other, induce oxidative stress by generating excess amount of reactive oxygen species (ROS).<sup>9</sup> The oxidative stress is known to impair ovarian functions.<sup>10</sup> Stress being an inevitable factor of life, cannot be avoided. Hence, measures should be taken to avoid the effects of stress despite experiencing stress. Since activation of the HPA axis is the root cause of the consequences of stress, the effects of stress can be alleviated by using the inhibitors of the synthesis or the receptor antagonists of HPA axis hormones viz., mifepristone, corticotrophin releasing hormone (CRH) antagonists, ketaconazole, metyrapone, etc. However, these synthetic compounds might have side effects. Therefore, the alternative for this is the use of naturally available time-tested herbs. *Withania somnifera* (ashwagandha) has been used extensively in the Ayurveda system of medicine since time immemorial.<sup>11</sup> Ashwagandha is commonly known as Indian winter cherry or Indian Ginseng.<sup>12</sup> Studies have reported that ashwagandha has several medicinal values viz., anti-diabetic, anti-inflammatory, hypoglycemic and antioxidant.<sup>13,14</sup> Further, ashwagandha also has anti-stress property.<sup>15</sup> However, the efficacy of this herb in the prevention of stress-induced alterations in the ovary is not studied. Further, a few studies have reported prevention of stress-induced alterations in the ovary by herbal extracts, i.e. ethanolic extract of *Symplocos racemosa* bark,<sup>16</sup> aqueous extract of *Rosa canina* hips,<sup>17</sup> and the ethanolic extract of *Euphorbia thymifolia* roots<sup>18</sup> in rats. These studies have used high doses (100 or 200 mg/kg body weight) of plant extracts and there is no comprehensive study to demonstrate alleviation of stress-induced alterations in gametogenic activity and antioxidant status of the ovary. Hence, the present study aims to find out whether, low doses of alcohol extract of the ashwagandha roots maintain normal ovarian activity in rats, despite the rats undergoing stress episodes.

## MATERIALS AND METHODS

### Experimental design

Adult female Wistar rats weighing about 170-190g with regular estrous cycles were procured from the central animal facility, University of Mysore, Mysuru. The rats had free access to food and water and were maintained under 12L/12D photoperiod. The experiment was approved by the Institutional animal ethics committee (Reference number : UOM/IAEC/17/2013, dated

28/09/2013) and the guidelines of the committee were followed for treatment of animals. The rats were randomly divided into four groups. The first group consisted of controls and were maintained without any disturbance. Each rat in the second group was intubated 1% carboxy methyl cellulose (CMC) (0.5ml/day). Each rat in the third group was exposed to stress regime every day, which consisted of exposure to restraint (1 h) followed by forced swimming (15 mins) after a gap of 4 hours (Grissom et al).<sup>19</sup> Rats in fourth group were exposed to stress regime similar to rats in 3<sup>rd</sup> group; however, alcoholic extract of ashwagandha (10 mg/kg body weight) was administered by oral intubation 1 hour prior to exposure to stress regime to each rat. The duration of the experiment was 4 weeks (28 days) and rats were sacrificed on the twenty ninth day. At autopsy, the blood sample, the ovary and the adrenal glands were collected and processed for the biochemical and histological analyses.

### Preparation of plant extract

The ashwagandha, *W. somnifera* was collected from the floral garden of the government college of Indian medicine, Mysuru and authenticated by an expert. The roots of ashwagandha were washed, shade dried and coarsely powdered. The powdered plant material was subjected to sequential extraction using soxhlet apparatus using solvents with increasing polarity, i.e., petroleum ether, benzene, chloroform, alcohol, water and NaOH. The alcoholic extract of the ashwagandha was found to have strong antioxidant property in our earlier study and hence it was used in the present experiment.

### Weight of the body, the adrenal gland and the ovary

The body weight of all rats was noted before the commencement of the experiment as initial body weight and at the autopsy as final body weight and based on these the percentage gain in body weight was computed. At autopsy, the weights of the ovary and the adrenal gland were also recorded and later converted to relative organ weight (weight per 100g body weight).

### Estrous cycle

The estrous cycle of each rat was studied following the procedure of Cooper et al.<sup>20</sup> Briefly, vaginal smear was taken daily and observed under microscope for the presence of different types of cells, based on which stage of the estrous cycle was determined. The average number of estrous cycles/ 4 weeks duration was computed.

### Serum hormone concentration

The serum concentrations of corticosterone and estradiol were estimated by enzyme-linked immunosorbent assay (ELISA) kits and the procedures provided by Demeditec diagnostics GmbH, Germany and Calbiotech, USA respectively.

### Biochemical analyses

The homogenate of the ovary and the adrenal gland was centrifuged at 2100 g. The supernatant was used to measure the activities of the adrenal and ovarian 3 $\beta$ -hydroxy steroid dehydrogenase (HSDH).<sup>21</sup> The activities of superoxide dismutase (SOD),<sup>22</sup> catalase (CAT),<sup>23</sup>

glutathione S- transferase (GST)<sup>24</sup> and glutathione peroxidase (GPx)<sup>25</sup> were determined using the homogenate of the ovary. The ovarian ferric reducing antioxidant power (FRAP)<sup>26</sup> and malondialdehyde (MDA)<sup>27</sup> concentration were also determined.

### Histology

Serial paraffin sections (5µm) of the ovary were cut and stained with haematoxylin and eosin. The antral follicles (380–740 µm size) were identified according to the description of Pederson and Peters<sup>28</sup> and atretic antral follicles were identified as per the description of Greenwald and Roy.<sup>29</sup> Each section of the ovary was focused and healthy and atretic antral follicles were separately counted. Further, care was taken to avoid counting the same follicle more than once. The aggregate of follicles in all the sections was expressed as the number of antral follicles/ovary/rat.

The active corpora lutea were identified based on the presence of active luteal cells and angiogenesis between them.<sup>30</sup> Focussing on each section of the ovary, the number of active corpora lutea was counted under 40X, and counting the same corpus luteum more than once was avoided.

### Granulosa cell apoptosis

The granulosa cells were isolated from the ovary<sup>31</sup> and were stained with ethidium bromide and acridine orange dye (1:1).<sup>32</sup> The cells were immediately observed under the fluorescent microscope. The cells emitting green fluorescence were categorized as healthy and those emitting yellow or orange fluorescence as apoptotic. The percentages of healthy and apoptotic granulosa cells were computed.

### DNA ladder assay

DNA was isolated from the ovary following the protocol of Compton.<sup>33</sup> It was subjected to separation on agarose gel electrophoresis. The changes, if any, were analyzed and photographed using gel doc.

## STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to find out the significant difference among different groups and judged significant if  $P < 0.05$ .

## RESULTS

### Weight of the body, weight and 3β-HSDH activity of the adrenal and serum concentration of corticosterone

There was a significant decrease in the percentage gain in the body weight in stressed rats compared to controls and vehicle controls. However, it was similar to that of controls in stressed rats treated with alcoholic extract of ashwagandha. (Table 1). The relative weight of the adrenal gland, activity of adrenal 3β-HSDH and the serum corticosterone concentration were significantly increased in rats exposed to stressors compared to controls, whereas that of vehicle treated controls and stressed rats treated with extract of ashwagandha did not significantly differ from controls (Table 1).

### Estrous cycle

The average number of estrous cycles in rats exposed to stressors was significantly decreased compared to controls, whereas that of stressed rats treated with ashwagandha extract did not significantly differ from controls (Table 2).

**Table 1**  
Effects of alcoholic extract of ashwagandha on the weight of body and the adrenal activity of rats

Groups & Treatments	% change in body weight (g)	Relative weight of adrenal gland (mg/100g bw)	Adrenal 3β-HSDH activity (nmol/mg/min)	Serum concentration of Corticosterone (ng/ml)
Control	12.74±1.72 <sup>b</sup>	30.83±0.70 <sup>a</sup>	0.23±0.02 <sup>a</sup>	128.75±21.25 <sup>a</sup>
Vehicle control	11.04±1.09 <sup>b</sup>	31.29±0.61 <sup>a</sup>	0.25±0.02 <sup>a</sup>	125.00±18.93 <sup>a</sup>
Stress	3.69±0.31 <sup>a</sup>	37.00±0.84 <sup>b</sup>	0.33±0.01 <sup>b</sup>	435.00±31.82 <sup>b</sup>
Stress+ashwagandha treatment (10mg/kg bw)	10.27±1.46 <sup>b</sup>	30.17±2.77 <sup>a</sup>	0.23±0.02 <sup>a</sup>	165.00±33.23 <sup>a</sup>
ANOVA	4.495	3.798	6.200	28.624
F-value	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.001$

Note: All the values are mean ± SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly ( $P < 0.05$ ) different as judged by DM RT.

**Table 2**  
Effects of alcoholic extract of ashwagandha on weight and 3β-HSDH activity of the ovary of rats

Groups	Relative weight of ovary (mg/100g body weight)	Average number of estrous cycles/4 weeks	Ovarian 3β-HSDH activity (nmol/mg/min)	Serum concentration of estradiol (pg/ml)
Control	53.50±1.87 <sup>b</sup>	6.00±0.33 <sup>b</sup>	0.209±0.02 <sup>b</sup>	31.42±1.07
Vehicle control	47.71±2.75 <sup>b</sup>	5.75±0.31 <sup>b</sup>	0.229±0.01 <sup>b</sup>	26.13±3.22
Stress	37.00±1.26 <sup>a</sup>	3.63±0.26 <sup>a</sup>	0.133±0.02 <sup>a</sup>	31.20±1.68
Stress+ ashwagandha treatment (10mg/kg bw)	48.25±2.23 <sup>b</sup>	5.50±0.33 <sup>b</sup>	0.186±0.02 <sup>a,b</sup>	27.30±3.99
ANOVA	8.183	12.267	5.072	0.469
F-value	$P < 0.003$	$P < 0.001$	$P < 0.05$	(NS)

Note: All the values are mean ± SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly ( $P < 0.05$ ) different as judged by DM RT.

**Weight and the activity of 3 $\beta$ -HSDH of ovary and serum estradiol concentration**

The relative weight of the ovary and ovarian 3 $\beta$ -HSDH activity in stressed rats were significantly lower when compared to controls, vehicle treated controls and ashwagandha extract treated stressed rats (Table 2). The serum concentration of estradiol did not differ significantly in rats of all the experimental groups (Table 2).

**Counts of antral follicles and corpora lutea**

The mean number of healthy antral follicles was significantly decreased and that of atretic antral follicles was significantly increased in stressed rats compared to those of controls and vehicle treated controls, whereas

those in stressed rats treated with alcoholic extract of ashwagandha did not differ from controls (Table 3). There was a significant decrease in the average number of active corpora lutea in stressed rats compared to controls, whereas stressed rats treated with ashwagandha extract did not differ from controls and vehicle controls (Table 3).

**Apoptosis of granulosa cells**

There was a significant reduction in the percentage of healthy granulosa cells, whereas that of apoptotic granulosa cells increased significantly in stressed rats compared to controls and extract treated stressed rats (Table 3).

**Table 3**  
**Effects of alcoholic extract of ashwagandha in the ovarian follicle development and apoptosis of the granulosa cells in rat**

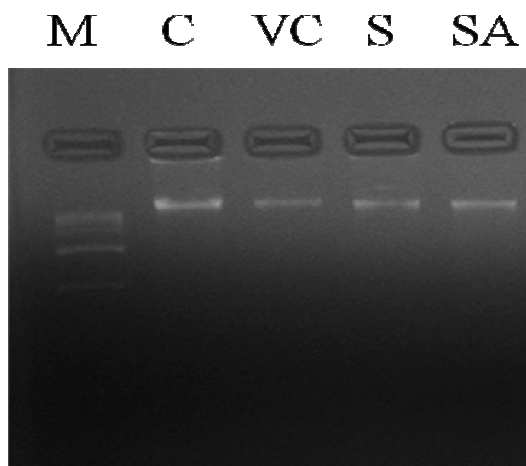
Groups & Treatments	Mean number of antral follicles $\pm$ SE		Mean number of active corpora lutea $\pm$ SE	Percentage of granulosa cells	
	Healthy	Atretic		Healthy	Apoptotic
Control	21.25 $\pm$ 0.85 <sup>b</sup>	9.50 $\pm$ 0.65 <sup>a</sup>	7.25 $\pm$ 0.25 <sup>b</sup>	36.69 $\pm$ 5.24 <sup>b</sup>	63.31 $\pm$ 6.19 <sup>a</sup>
Vehicle control	24.00 $\pm$ 1.58 <sup>b</sup>	8.50 $\pm$ 1.32 <sup>a</sup>	8.00 $\pm$ 1.08 <sup>b</sup>	30.93 $\pm$ 1.86 <sup>b</sup>	69.07 $\pm$ 1.86 <sup>a</sup>
Stress	14.50 $\pm$ 1.32 <sup>a</sup>	15.00 $\pm$ 1.63 <sup>b</sup>	3.50 $\pm$ 0.29 <sup>a</sup>	16.51 $\pm$ 1.23 <sup>a</sup>	83.49 $\pm$ 1.23 <sup>b</sup>
Stress + ashwagandha treatment (10mg/kg bw)	22.75 $\pm$ 1.65 <sup>b</sup>	6.50 $\pm$ 1.32 <sup>a</sup>	8.25 $\pm$ 0.48 <sup>b</sup>	32.19 $\pm$ 3.90 <sup>b</sup>	67.81 $\pm$ 3.90 <sup>a</sup>
ANOVA	9.308	8.038	12.649	5.043	5.209
F- Value	P<0.003	P<0.05	P<0.05	P<0.05	P<0.05

Note: All the values are mean  $\pm$  SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by DM RT.

**DNA ladder assay**

No significant difference was found in the electrophoretic profile of ovarian DNA among rats of different experimental groups (Fig 1).



**Figure 1**  
**Photograph of gel electrophoretic profile of DNA from the ovaries of rats of different groups.**

Note the similar pattern in the electrophoretic profile of DNA among controls and stressed and plant extract treated stressed rats. M- Marker, C-Control, S-Stress, SA – Stressed rats pre-treated with 10 mg/kg body weight of ethanolic extract of ashwagandha and VC – Control rats treated with 0.5 ml of vehicle (1% carboxy methyl cellulose).

**Antioxidant status of the ovary**

The activities of the SOD, CAT, GST and GPx and the FRAP value were decreased significantly in stressed rats compared to controls, whereas those of ashwagandha extract treated stressed rats did not differ from controls (Table 4).

Table 4

Effects of alcoholic extract of ashwagandha on the activities of antioxidant enzymes, ferrous reducing antioxidant power (FRAP) and concentration of malondialdehyde of the ovary in rat

Groups	SOD (U/mg protein)	CAT (nmol/mg/min)	GST ( $\mu$ mol/mg/min)	GPx ( $\mu$ mol/mg/min)	FRAP ( $\mu$ mol/l FeSO <sub>4</sub> )	MDA concentration (nmol/mg protein)
Control	0.068 $\pm$ 0.01 <sup>b</sup>	0.0015 $\pm$ 0.00 <sup>b</sup>	200.19 $\pm$ 18.80 <sup>b</sup>	0.013 $\pm$ 0.00 <sup>b</sup>	405.23 $\pm$ 51.55 <sup>b</sup>	211.38 $\pm$ 28.40 <sup>a</sup>
Vehicle control	0.065 $\pm$ 0.01 <sup>b</sup>	0.0014 $\pm$ 0.00 <sup>b</sup>	173.44 $\pm$ 21.97 <sup>b</sup>	0.015 $\pm$ 0.00 <sup>b</sup>	395.08 $\pm$ 42.57 <sup>b</sup>	220.84 $\pm$ 17.98 <sup>a</sup>
Stress	0.012 $\pm$ 0.00 <sup>a</sup>	0.0004 $\pm$ 0.00 <sup>a</sup>	72.26 $\pm$ 17.92 <sup>a</sup>	0.004 $\pm$ 0.00 <sup>a</sup>	183.69 $\pm$ 18.08 <sup>a</sup>	466.84 $\pm$ 45.13 <sup>b</sup>
Stress ashwagandha treatment (10mg/ kg bw)	0.054 $\pm$ 0.01 <sup>b</sup>	0.013 $\pm$ 0.00 <sup>b</sup>	225.39 $\pm$ 19.28 <sup>b</sup>	0.013 $\pm$ 0.00 <sup>b</sup>	429.23 $\pm$ 55.44 <sup>b</sup>	294.14 $\pm$ 26.12 <sup>a</sup>
ANOVA	32.321	54.821	11.778	9.022	6.604	14.543
F-Value	P<0.001	P<0.001	P<0.003	P<0.001	P<0.05	P<0.001

Note: All the values are mean  $\pm$  SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by DM RT.

### MDA concentration

There was a significant increase in the concentration of MDA in stressed rats compared to controls and stressed rats treated with ashwagandha (Table 4).

## DISCUSSION

It is known that stress activates the HPA axis leading to an increase in the secretion of glucocorticoids.<sup>7</sup> In the present study, an increase in the adrenocortical activity as shown by an increase in the relative weight and the 3 $\beta$ -HSDH activity of the adrenal gland coupled with a significantly higher serum corticosterone concentration indicates the activation of the HPA axis following exposure to stressors for 4 weeks. In addition, the response of the adrenal gland, even after daily exposure to stressors for 4 weeks indicates that rats exhibited stress response without getting habituated to stress regime. Further, concomitant with the activation of adrenocortical activity, the gametogenic activity and antioxidant status of the ovary were altered as shown by a decrease in the number of healthy antral follicles with concomitant increase in atretic antral follicles and a reduction in antioxidant potential of the ovary. Hence, these were ovarian stress responses. Interestingly, these stress-induced alterations were not observed in stressed rats pre-treated with alcoholic extract of ashwagandha root. This study probes into possible mode of alleviation of stress-induced ovarian dysfunction. Stress is known to adversely affect different ovarian functions. For instance, stress impairs the developmental potential of oocyte<sup>5,34,35</sup> and follicular development,<sup>3,4</sup> blocks ovulation<sup>6</sup> and reduces steroidogenesis.<sup>7</sup> Similarly, in this study exposure of rats to stressors decreased the ovary weight and 3 $\beta$ -HSDH activity, the number of healthy follicles, percentage of healthy granulosa cells, number of corpora lutea, activities of antioxidant enzymes with concomitant increase in the mean number of atretic follicles, the percentage of apoptotic granulosa cells and MDA concentration. Chronic stress-induced alterations in the ovary may be due to suppression of the HPG axis by the activated HPA axis,<sup>7</sup> because the hormones of the HPA axis are known to impair secretion or action of gonadotropins, which regulate the ovarian activity. For

instance, CRH suppresses the gonadotropin releasing hormone (GnRH) secretion<sup>36</sup> and cortisol suppresses GnRH secretion leading to inhibition of the luteinizing hormone pulse frequency during the follicular phase of ewes<sup>37</sup> and sheep.<sup>6</sup> GC, the ultimate product of activated HPA axis is also known to directly affect the functions of the ovary by reducing luteinising hormone receptors on granulosa cells<sup>38</sup> or the steroidogenic activity,<sup>39</sup> or gametogenic activity.<sup>7</sup> In addition, GC is also known to induce oxidative stress by generating enormous amounts of ROS.<sup>9</sup> Though a few studies reported stress-induced oxidative damage in the ovary of rats,<sup>16,18,34</sup> its impact on ovarian components has not been studied, excepting demonstration of decrease in developmental potential of oocyte.<sup>34</sup> However, impact of ROS, generated either by chemical treatment or pathological states on the ovary is studied extensively.<sup>40</sup> For instance, an increase in ROS in the ovary results in apoptosis of the granulosa and theca cells,<sup>41</sup> impairment in oocyte developmental potential,<sup>42</sup> and decrease in the steroidogenic capacity of the ovary.<sup>10</sup> The present study also demonstrates the oxidative damage following stress exposure as there was an increase in the ovarian MDA concentration coupled with a decrease in antioxidant enzyme activities and its impact on ovarian activity as shown by an increase in granulosa cell apoptosis and antral follicle atresia. Hence, stress-induced alterations in the ovary may be synergistic effects of altered HPG axis hormones secretion/action and oxidative stress. Therefore, these two aspects have to be considered in designing treatments to prevent stress effects on the ovary. Since stress is an inescapable fact of life in this competitive society and adversely affects ovarian functions, efforts to be made to prevent the stress-induced ovarian dysfunction. It is clear from the above discussion that activation of the HPA axis is the root cause of the stress effects. Hence, prevention of HPA axis activation under stressful condition may prevent the stress effects. This can be achieved by treatment with the inhibitors/antagonists of HPA axis hormones viz., mifepristone (receptor antagonist of glucocorticoid), ketaconazole, metyrapone (synthesis inhibitors of glucocorticoid), antalarmin (CRH type-1 receptor antagonist),  $\alpha$ -helical CRH (CRH receptor antagonist), etc. Prevention of stress effects by

mifepristone,<sup>43</sup>  $\alpha$ -helical CRH,<sup>44,3</sup> ketaconazole<sup>45</sup> and metyrapone<sup>44</sup> have been reported. However, these synthetic drugs have side effects on long term usage. For instance, mifepristone, cannot be suggested for women as anti-stress agent since it also has antiprogesterone property. It is also known to block ovulation when administered just before the luteinizing hormone surge.<sup>46</sup> Further, ketaconazole and metyrapone if not used in low doses may cause adrenal insufficiency.<sup>47</sup> Hence, herbal products which have been used in traditional medicine for years would be a better alternative, as these have no or little side effects. In this regard ashwagandha, widely used herb with several beneficial properties has great potential.<sup>13,14</sup> It is interesting to note that, the rats pretreated with extract of ashwagandha and exposed to stressors, did not exhibit adrenal stress response, as weight and the steroidogenic enzyme activity ( $3\beta$ -HSDH) of the adrenal gland and serum concentration of corticosterone did not differ from controls, whereas all these parameters were significantly lower than stressed rats. Therefore, despite undergoing stress episodes, there was no activation of the HPA axis. Thus, the implications of activated HPA axis due to stress could be prevented by this extract. Indeed, the ovary of rats pre-treated with the extract and then exposed to stress, did not show disruption of ovarian activities, i.e., increase in follicular atresia and apoptosis of granulosa cells and a decrease in ovarian  $3\beta$ -HSDH activity and number of corpora lutea as found in stressed rats. Thus, our results, for the first time demonstrate that, ashwagandha extract is potent enough to rescue the ovary from deleterious effects of stress. Further, the normal ovarian activity in extract treated stressed rats was also reflected in the reproductive cycle as these rats showed regular estrous cycles unlike disrupted cycles of stressed rats. This study also reveals that this effect of ashwagandha is due to their ability to suppress HPA axis activation, because the serum concentration of corticosterone in stressed rats treated with the herbal extracts was similar to controls. Since high levels of glucocorticoid disrupts ovarian activity as discussed above, the prevention of hypersecretion of corticosterone in herbal extract treated stressed rats, has rescued the ovary from detrimental effect of stress. In addition, the herbal extract has also prevented the oxidative damage because the antioxidant enzyme activities as well as MDA concentrations were similar to controls in the ovaries of extract treated stressed rats. Thus, our present study clearly demonstrates that the extract of ashwagandha rescue the ovary from deleterious effects of stress by preventing HPA axis activation as well as oxidative damage. These results have far reaching consequence, as many women suffer from ovarian dysfunction due to stress resulting in infertility,<sup>48</sup> impairment in follicular maturation, ovulation and implantation<sup>49</sup> and hasty depletion of ovarian reserves.<sup>50</sup> In addition, in our earlier study with a similar stress regime for a longer duration of 12 weeks in rats, resulted in a pathophysiological condition, polycystic ovary phenotype, suggesting the severity of stress effects.<sup>51</sup> However, our present study demonstrates possibility of prevention of severe consequences of stress by a widely used medicinal plant. In our earlier study, pretreatment with a single dose (10 mg/kg body weight) of alcoholic extract of

ashwagandha<sup>52</sup>, prevented stress-induced hyperglycemia throughout the day in rats. Thus, a single dose is sufficient to protect the deleterious effect of stress for whole day in rats. Therefore, ashwagandha extract is a promising candidate for developing drugs to prevent stress effects on reproduction. Earlier, a few studies have reported the prevention of stress-induced alterations in the ovary by other herbal extracts. For instance prevention of cold restraint induced oxidative damage, disruption of the estrous cycle, decrease in the weight of ovary and histomorphology of the ovary by an ethanolic extract of *Symplocos racemosa* bark,<sup>16</sup> restraint induced disruption of the estrous cycle and weights of ovary, uterus and adrenal gland by aqueous extract of *Rosa canina* hips<sup>17</sup> and restraint induced oxidative damage by an ethanolic extract of *Euphorbia thymifolia* roots<sup>18</sup> have been reported. Despite, these studies, our study gains importance since the dose of alcoholic extract of ashwagandha used was 10 mg / kg body weight, which is 10 fold lesser than the dose used in the earlier similar studies. Yet this was effective enough to prevent the activation of the HPA axis. In addition, the present work is the first comprehensive study showing the efficacy of herbal extract in the prevention of stress-induced alterations in gametogenic and antioxidant status of the ovary. In recent years, though isolated compounds from the herbal extracts are the chief interests of the pharmaceutical companies in the formulation of synthetic drugs, the herbal crude extracts are of interest for various reasons. First and foremost advantage of using herbal crude extracts is that it is cost effective and easily available, whereas pharmaceutically designed pure drugs (based on the isolated compound from the extract) are expensive and poor people in the remote areas who are in actual need, cannot afford it.<sup>53</sup> Further, the active constituent may get degraded during fractionation which in turn may reduce the activity of the compound.<sup>54</sup> Furthermore, a synergistic effect can be produced when different constituents of an extract interact with each other. In addition, different constituents of a monoextract may affect different targets and thus improving the condition of an individual in a synergistic way.<sup>55</sup>

## CONCLUSION

The present study shows the efficacy of alcoholic extract of ashwagandha root in maintaining the normal ovarian functions despite stress experienced by the rats. The present study also demonstrates that crude extract of commonly available herb, has great potential to be developed as anti-stress drugs.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

## REFERENCES

- Bhattacharya K, Ghosal S. Experimental evaluation of the antistress activity of a herbal formulation, zetress. J Nat Remedies 2000;1:17.
- Chatterjee A, Rajikin MH, Chatterjee R, Ghosh S. Mini review: Stress and how it affects reproduction. Biomed Res 2006;17(1):1-6.
- Bhat MS, Yajurvedi HN. Stress induced alterations in pre-pubertal ovarian follicular development in rat. J Stress Physiol Biochem 2011;7(4):51-68.
- Wu LM, Liu YS, Tong XH, Shen N, Jin RT, Han H, et al. Inhibition of follicular development induced by chronic unpredictable stress is associated with growth and differentiation factor 9 and gonadotropin in Mice. Biol Reprod 2011;86(4):121.
- Zhang SY, Wang JZ, Li JJ, Wei DL, Sui HS, Zhang ZH, Zhou P, Tan JH. Maternal restraint stress diminishes the developmental potential of oocytes. Biol Reprod 2011;84:672-81.
- Daley CA, Macfarlane MS, Sakurai H, Adams TE. Effect of stress-like concentrations of cortisol on follicular development and the preovulatory surge of luteinizing hormone in sheep. J Reprod Fertil 1999;117:11-6.
- Whirledge S, Cidlowski JA. Glucocorticoid, stress, and fertility. Minerva Endocrinol 2010;35:109-25.
- Chrousos GP, Torpy DJ, Gold PW. Interactions between the hypothalamic-pituitary-adrenal axis and the female reproductive system: clinical implications. Ann Intern Med 1998;129:229-40.
- Spiers JG, Chen HC, Sernia C, Lavidis NA. Activation of the hypothalamic-pituitary-adrenal stress axis induces cellular oxidative stress. Front Neurosci 2015;8:456
- Agarwal A, Gupta S, Sikkab S. The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol 2006;18:325-2.
- Singh N, Bhalla M, de Jager P, Gilca M. An overview on ashwagandha: a rasayana (rejuvenator) of ayurveda. Afr J Tradit Complement Altern Med 2011;8(S):208-213.
- Bhatnagar M, Sisodia SS, Bhatnagar R. Antulcer and antioxidant activity of *Asparagus racemosus* WILD and *Withania somnifera* DUNN in rats. Ann NY Acad Sci 2005;1056:261-78.
- Mishra L-C and Singh BB. Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha): A review. Altern Med Rev 2000;5(4):334-46.
- Ahlawat P, Khajuria A, Bhagwat DP, Kalia B. Therapeutic benefits of *Withania somnifera*: An exhaustive review. Int j pharm chem sci. 2012;1(2):491-6.
- Bhattacharya SK, Bhattacharya A, Chakrabarti A. Adaptogenic activity of Siotone, a polyherbal ayurvedic rasayana formulation. Ind J Exp Biol 2000;38:119-128.
- Saraswathi CD, Gupta SK, Sreemantula S. Protective effect of *Symplocos racemosa* bark on cold restraint stress induced reproductive changes in female rats. J Nat Prod. 2012;5:251-58.
- Gupta SK, Gupta A, Prakash D, Gupta AK, Vedpal. Protective effect of *Rosa canina* L. on stress induced reproductive changes in female rats. IRJP 2013;4(2):84-6.
- Gudipudi S, Puranik DS, Alla R, Ajarapu U, Kistammagari RT. Role of *Euphorbia thymifolia* L. ethanolic root extract in treating female reproductive dysfunction in rats. IJPR 2015;5(4):doi:10.7439/ijpr
- Grissom N, Kerr W, Bhatnagar S. Struggling behavior during restraint is regulated by stress experience. Behav Brain Res 2008;191:219-26.
- Cooper RL, Goldman JM, Vandenberg JG. Monitoring of the estrous cycle in the laboratory rodent by vaginal lavage. In: Heindel JJ and Chapin RE, eds. Methods in Toxicology: Female Reproductive Toxicology. San Diego: Academic Press; 1993. p. 45-56.
- Shivanandappa T, Venkatesh S. A colorimetric assay method for 3beta-hydroxy delta-5-steroid dehydrogenase. Anal Biochem 1997;254:57-61.
- Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for SOD. Eur J Biochem 1974;47:469-73.
- Aebi H. Catalase *in vitro* methods. Method Enzymol 1984;105:121-6.
- Habig WJ, Babst MJ, Jacoby WJ. Glutathione -S-transferase the 1<sup>st</sup> enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130-39.
- Tappel AL. GPx and hydroperoxidase. Methods Enzymol 1978;52:506-13.
- Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239:70-6.
- Ohkawa H, Ohishi N, Yogi K. Assay for LPO in animal tissue by thiobarbituric acid reaction. Anal Biochem 1979;95:351-58.
- Pedersen T, Peters H. Proposal for a classification of oocytes and follicles in the mouse ovary. J Reprod Fertil 1968;17:555-57.
- Greenwald GS, Roy SJ. Follicular development and its control. In Knobil E and Neil JD, eds. "Physiology of Reproduction". New York: Raven Press Ltd; edn 2, 1994. P. 629-724.
- Yoshida M, Sanbuissyo A, Hisada S, Takahashi M, Ohno Y, Nishikawa A. Morphological characterization of ovary under normal cycling in rats and its viewpoints of ovarian toxicity detection. J Toxicol Sci 2009;34:SP189- SP97.
- Campbell KL. Ovarian granulosa cells isolated with EGTA and hypertonic sucrose: Cellular integrity and function. Biol Reprod 1979;21:773-86.
- Kasibhatla S, Amarante-Mendes GP, Finucane D, Brunner T, Bossy-Wetzel E, Green DR. Acridine orange/Ethidium bromide (AO/EB) staining to detect apoptosis. Cold Spring Harbor Protocols 2006; 2006(3) pii: pdb.prot4493.
- Compton MM. A biochemical hallmark of apoptosis: Internucleosomal degradation of the genome. Cancer and Metastasis Rev 1992;11:105-19.

34. Lian HY, Gao Y, Jiao GZ, Sun MJ, Wu XF, Wang TY, Li H, Tan JH. Antioxidant supplementation overcomes the deleterious effects of maternal restraint stress-induced oxidative stress on mouse oocytes. *Reproduction* 2013;146:559-68.
35. Gao Y, Chen F, Kong QQ, Ning SF, Yuan HJ, Lian HY, et al. Stresses on female mice impair oocyte developmental potential: Effects of stress severity and duration on oocytes at the growing follicle stage. *Reprod Sci* 2016;23(9):1148-57.
36. Kyrou I, Tsigos C. Chronic stress, visceral obesity and gonadal dysfunction. *Hormones* 2008;7:287-93.
37. Oakley AE, Breen KM, Clarke IJ, Karsch FJ, Wagenmaker ER, Tilbrook AJ. Cortisol reduces gonadotropin-releasing hormone pulse frequency in follicular phase Ewes: Influence of ovarian steroids. *Endocrinol* 2009;150(1):341-49.
38. Kawate N, Inaba T, Mori J. Effects of cortisol on the amounts of estradiol-17 $\beta$  and progesterone secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. *Anim Reprod Sci* 1993;32:15-25.
39. Wolfenson D, Lew BJ, Thatcher WW, Graber Y, Meidan R. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. *Anim Reprod Sci* 1997;47:9-19.
40. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. *Reprod Biol Endocrinol* 2012;10:49.
41. Devine PJ, Perreault SD, Ludere U. Roles of Reactive Oxygen Species and Antioxidants in ovarian toxicity. *Biol Reprod* 2012;86(2):27.
42. Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001;7(2):175-89.
43. Nirupama M, Yajurvedi HN. Efficacy of ashwagandha (*Withania somnifera* L.) root extracts in preventing stress-induced testicular damage in rat. *European J Biomed Pharm sci* 2015;2(7):413-24.
44. Madhuranath BN, Yajurvedi HN. Progesterone prevents corticosterone mediated inhibition of estrous behavior in rats. *Indian J Exp Biol* 2011;49:313-18.
45. Cohen H, Benjamin J, Kaplan Z, Kotler M. Administration of high-dose ketoconazole, an inhibitor of steroid synthesis, prevents posttraumatic anxiety in an animal model. *Eur Neuropsychopharmacol* 2000;10:429-35.
46. Brannstrom M. Inhibitory effect of mifepristone (RU 486) on ovulation in the isolated perfused rat ovary. *Contraception* 1993;48(4):393-402.
47. Goeders NE, Guerin GF. Effects of the combination of metyrapone and oxazepam on cocaine and food self-administration in rats. *Pharmacol Biochem Behav* 2008;91(1):181-9.
48. Sanders KA, Bruce MW. A prospective study of psychosocial stress and fertility in women. *Hum reprod* 1997;12(10):2324-29.
49. Nepomnaschy PA, Welch K, McConnell D, Strassmann BI, England BG. Stress and female reproductive function: A study of daily variations in cortisol, gonadotrophins and gonadal steroids in a rural mayan population. *Am J Hum Biol* 2004;16:523-32.
50. Bleil ME, Adler NE, Pasch LA, Sternfeld B, Gregorich SE, Rosen MP, Cedars MI. Psychological stress and reproductive aging among pre-menopausal women. *Hum Reprod* 2012;27(9):2720-8.
51. Divyashree S, Yajurvedi HN. Long-term chronic stress exposure induces PCO phenotype in rat. *Reproduction* 2016; 152: 765-774.
52. Nirupama R, Devaki M, Nirupama M, Yajurvedi HN. In vitro and in vivo studies on the hypoglycaemic potential of ashwagandha (*Withania somnifera*) root. *Pharma science monitor* 2014;5(3):45-58.
53. Hirt H, M'Pia B. *Natural Medicine in the Tropics I: Foundation Text*. Winnenden: Anamed; 2008.
54. Rasoanaivo P, Wright CW, Willcox ML, Gilbert B. Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. *Malar J* 2011;10:S4
55. Wagner H, Ulrich-Merzenich G. Synergy research: Approaching a new generation of phytopharmaceuticals. *Phytomedicine* 2009;16:97-110.