

Protective effect of *Symplocos racemosa* bark on cold restraint stress induced reproductive changes in female rats

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ABSTRACT

Here we evaluate the ethanolic extract of *Symplocos racemosa* bark in treating female reproductive dysfunctions. Cold restraint stress (4°C for 3h/day for 28 days) was used as stressor to induced changes in reproductive dysfunctions. Rats were pretreated with 100mg/kg body weight and 200mg/kg b.w. for 15 days with ethanolic extract of *Symplocos racemosa* bark and were continued for next 28 days along with induction of stress. Assessment of its effectiveness was done by observing changes in estrous cycle, biochemical parameters, antioxidant enzyme estimations and histopathological studies. *Symplocos racemosa* treated rats showed significant changes in the different phases of estrous cycle and restored to normal. Improvement in the weight of ovaries, uteri, liver and decrease in the weight of adrenal glands were also seen. Serum glucose, cholesterol, triglycerides and SOD, catalase enzyme levels in ovary and uterus showed prevention of fall, and decrease in the lipid peroxidation of ovary and uterus when compared with stressed control. From the experimental studies, ethanolic extract of *Symplocos racemosa* bark at two different doses showed promising improvement in treating female reproductive dysfunctions induced by cold restraint stress. The above activity may be due to the presence of various secondary metabolites like alkaloids, flavonoids, phytosterols and other constituents present the in ethanolic extract of *Symplocos racemosa* bark.

Keywords: *Symplocos racemosa*; Cold restraint stress; Estrous cycle; Antioxidant.

INTRODUCTION

Reproductive failure is a significant concern in both developed and developing countries because of its high importance in every family (Ruder, et al., 2008) Infertility is defined as the failure to conceive a recognized pregnancy after 12 months of unprotected intercourse. Among such couples, causative factors are found in about 30-40% in females, 10-30% in males and in 15-30% of cases, both partners have detectable abnormalities (Marcia, 2003).

High stress perception is a risk factor for PCOS, anovulation, severe premenstrual pain, pregnancy outcomes including preterm delivery and low birth weight, as well as postpartum depression and early onset of perimenopause (Greenberg, 2002). All of the above causes are due to the suppression of gonadotrophic hormones, activation of sympathoadrenomedullary system and oxidative stress (Nakamura, 2008).

Symplocos racemosa Roxb. (Family-*Symplocaceae*) commonly known as lodh or lodhra, widely used as a female fertility improving drug (Chopra, et al., 1994). It is a small tree found throughout India. Traditionally the bark is widely used in the treatment of hemorrhage, eye diseases, spongy and bleeding gums, wounds, ulcers, tumors, leprosy, skin diseases, asthma, bronchitis, dropsy, arthritis, fever, menorrhagia, uterine disorders, leucorrhea, aphrodisiac, cancer, diarrhoea, dysentery, and bowel complaints (Gupta, 2010). Previous studies have used ethanolic extract for studying various activities such as antipyretic (Vijayabaskaran, et al., 2010), hepatoprotective, antitumor, antimicrobial, antiulcer, etc. Present study focus for the first time on stress induced reproductive changes in rats. Hence ethanolic extract of *S. racemosa* bark, was selected for female fertility improving activity in stressed rats.

MATERIALS AND METHODS

Plant: The fresh bark of *S. racemosa* was collected from Shimoga district, Karnataka, in December 2010, identified by Dr. N Shiddamallayya, National Ayurveda Dietetics Research Institute, Bangalore, with voucher number RRCBI/MCW/O3.

Preparation of ethanolic extract: The bark of *S. racemosa* was chopped into small pieces and dried under shade at room temperature. The dried bark was powdered and passed through coarse sieve (10/44). The dried, powdered of *Symplocos racemosa* bark (200g) was extracted with 95% (V/V) ethanol (500ml) for 20h in a Soxhlet extractor till complete exhaustion. Ethanolic extract was concentrated at 40°C to obtain dark brown residue. Yield was 10% w/w with respect to dried power. Preliminary phytochemical studies showed the presence of carbohydrates, glycosides, alkaloids, saponins, terpenoids, flavonoids and phytosterols (Devmurari, 2010)

Animals: Experimental study was carried out using adult female Wistar Albino rats weighing between 170-200g. The animals were procured from Drug Testing Laboratory, Bangalore. The animals were housed in polypropylene cages. The cages were maintained clean and hygienic. Animals were acclimatized in light and temperature controlled room with a 12-12h dark-light cycle, temperature 25±2°C and humidity 50±5%. The rats were fed with commercial pelleted rat feed and water *ad libitum*. The animal caring and handling were done according to the CPCSEA guidelines. The Institutional Animal Ethics Committee (IAEC/NCP/16/10) dated 24/12/2010 at Nargund College of Pharmacy has approved the study.

Dose selection: Two doses (100mg/Kg and 200mg/Kg) of ethanolic extract of *S. racemosa* bark (EESR) were selected as reported by Vijayabaskaran, et al., 2010.

Cold restraint stress model: Animals with regular estrous cycle were selected and divided into four groups, six animals in each group. Animals were individually placed in a plastic restrainer (21cm in length x 6cm in diameter) with ventilated sliding doors and then placed in a cold chamber at 4°C for 3h/day for 28 days (Dhanalakshmi, et al., 2006; Marcelo, et al., 2008; Saraswathi, et al., 2010).

Group I- Vehicle control - distilled water, orally (5ml/kg body weight) for 28 days.

Group II- Cold restraint stress (CRS) (4°C for 3h/day) for 28 days.

Group III and IV- Rats were pretreated with ethanolic extract of *S. racemosa* bark (100mg/kg b.w., p.o. and 200mg/kg b.w., p.o. respectively) for 15 days prior to the starting of cold restraint stress and was continued for another 28 days along with induction of stress from 16th day.

Group III, IV animals were subjected to cold restraint stress at 4°C for 3h/day after half an hour of administration of the ethanolic extract of *S. racemosa* bark extract.

Every day, immediately after the stress session, vaginal smears were examined in all the groups (Turner, 1971). After the last stress session on 28th day blood was collected from all the groups by puncturing retro-orbital plexus. Serum was separated. Serum glucose, cholesterol and triglyceride levels were estimated by semi auto analyzer (Robonik prietest). Kits were supplied by PRISM diagnostics Ltd. Immediately after the blood collection, all animals were sacrificed by cervical dislocation. Liver, ovaries, uteri, adrenal glands were isolated and weighed. One ovary and uterus were placed in KCl (10% w/v) solution and homogenized by homogenizer and centrifuged at 4000rpm for 10 min. The tissue homogenate was estimated for lipid peroxidation (Ohkawa, et al., 1979). The supernatant was separated and estimated for SOD (Marklund and Marklund, 1972), catalase (Sinha, 1972) and protein estimation was done by the method of Lowry et al., 1951. Another ovary and uterus were preserved in 15% buffered formalin solution for histopathological studies.

RESULTS

Stressed animals showed significant changes ($P<0.001$) in estrous cycle when compared to vehicle control. Prior and continued treatment with ethanolic extract at both doses of ESSR along with application of stress, the change in the different phases of estrous cycle were less compared to CRS group (Table 1).

Table 2 showed significant changes ($P<0.001$) in the organ weights of stressed rats. Animals pretreated with ESSR at both doses reduced stress induced changes significantly.

A significant decrease ($P<0.001$) in the level of serum glucose, cholesterol and triglycerides was seen in stressed rats. Animals pretreated with ESSR showed significant prevention of fall ($P<0.001$) in the levels of serum glucose, cholesterol and triglycerides when compared with the CRS group (Table 3).

Table 4 showed a significant decrease ($P<0.001$) in the enzyme levels of superoxide dismutase, catalase and increase ($P<0.001$) in lipid peroxidation level in both uterus and ovary in stressed rats. Pretreatment with ESSR showed significant prevention in the fall ($P<0.001$) of enzyme levels of superoxide dismutase, catalase and also ($P<0.001$) lipid peroxidation in both uterus and ovary.

DISCUSSION

Cold restraint stress produces psychological and physiological stress and was chosen as stress inducer to produce female reproductive dysfunctions (Saraswathi, et al., 2010). Ethanolic extract of *Symplocos racemosa* bark was evaluated for female fertility improving activity in stressed rats using cold restraint stress models.

The estrous cycle in rats involves many histological, physiological and morphological and biochemical changes within the ovary. During the estrous cycle the maturation and ovulation of preovulatory follicles takes place under the combined and balanced influence of ovarian and extra ovarian hormones. Any imbalance in these hormones leads to irregularity in the function of ovary and changes in the duration of estrous cycle (Koneri, et al., 2006). Similarly oxidative stress also causes

damage during oocyte maturation and ovulation (Agarwal, et al., 2005).

Cold restraint stressed rats showed a significant increase in the mean number of days in proestrous phase and decrease in estrous, metestrous and diestrous phases indicating the arrest of follicular development at the initial stages leading to non-maturation of the follicles. Non availability of matured follicle indicates decrease estrous and metestrous phases. This disruption in the growth and differentiation of preovulatory follicles may be due to the non-availability of steroidal hormones, which are essential for their maturation and differentiation; local estrogen produced by granulosa cells, imbalanced endogenous steroid and protein hormones or due to the generation of free radicals likes Reactive Oxygen Species (ROS). *Symplocos racemosa* treated groups showed significant decrease in the mean days of proestrous phase which indicates the development of follicles and significant increase in the mean days of estrous, metestrous and diestrous phases when compared with cold restraint stressed rats showing antagonizing effect against stress. It also indicates the maturation of the follicles, formation of Graafian follicles and corpus luteum which may be due to the increased secretion of either gonadotrophic or steroidal hormones or both or reversal of the stress induced oxidative stress (Bhutani, et al., 2004).

Ovaries are considered to be an aggregate of three endocrine tissues, the stroma, the follicle and the corpus luteum. The weight of these tissues constitutes the net weight of ovaries. The decrease in ovarian weights in stressed rats clearly indicated that there was no development of the follicles and hence decreases in activity of stroma, follicle and corpus luteum due to non-availability of either gonadotrophic or steroidal hormones or both (Shivalinagappa, et al., 2002) or due to oxidative stress. *Symplocos racemosa* treated groups showed a significant prevention in the loss of weight of ovaries which may be due to the availability of gonadotrophic or steroidal hormones or due to combating oxidative stress.

A significant decrease in uterine weight was also seen in stressed rats due to the non-availability of the hormones required for the development of uterus. *Symplocos racemosa* treated groups showed a significant prevention in the loss of weight of uterus which may be due to uterotrophic effect.

The increase in weight of adrenal glands in stressed rats may be due to the active involvement of the Hypothalamic Pituitary Adrenal (HPA) axis and sympathetic activation, which is highly responsive to stress. The adrenal hypertrophy takes place in response to the secretion of Adrenocorticotrophic Hormone from the pituitary for increased corticosterone from cortical cells to combat stress (Kenjale, et al., 2007). *S. racemosa* treated groups showed significant decrease in the weight of adrenal glands which may be due to reversal of stress-induced adrenomedullary response to decreased production of corticotropic hormone (Kenjale, et al., 2007).

Chronic stress depletes the energy stores resulting in decreased serum glucose, cholesterol and triglyceride levels. The same was seen in stressed rats which may be due to redirection of glucose to the specific stress demanding sites and depletion of stored glycogen (Singh, et al., 2001; Kioukia, et al., 2002). Glucose also is a major non-enzymatic antioxidant and scavenges hydroxyl radicals. Therefore the decreased level of serum glucose causes activation of ROS family (Ayesha and Naheed, 2009). Utilization of reserve fats as a secondary substrate in response to corticosterone, leads to decrease in the level of triglycerides and cholesterol. The reduction in serum triglycerides may be secondary to the effect of catecholamines on the triglyceride lipase activity in the adipose tissue (Kioukia, et al., 2002; Saraswathi, et al., 2010).

S. racemosa treated groups showed significant prevention in the fall of serum glucose, cholesterol and triglycerides levels indicating antagonism to stress induced changes. The results were similar to the previous studies done by Kannur et al., 2006.

SOD is the first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2). CAT is a peroxisomal haem protein that catalyses the removal of H_2O_2 formed during the reaction catalysed by SOD. Thus, SOD and CAT acts mutually supportive antioxidative enzymes, which provide protective defense against ROS. These ROS are very unstable and highly reactive. They become stable by acquiring electron from nucleic acids, proteins, carbohydrates and lipids there by cascade of chain reaction are initiated resulting in cellular damage and causes Lipid Peroxidation (LPO) (Vijayabaskaran, et al., 2010). Ovaries and uterus of stressed rats showed a significant decrease in the SOD, catalase enzyme activities due to the excessive generation of free radicals. The results were similar to other findings (Davydov, et al., 2004) suggesting that stress stimulates free radical generation in the liver of rats, and increased LPO resulting in cellular damage in stressed rats. Malondialdehyde a secondary product of lipid peroxidation is a major reactive aldehyde, higher levels can lead to peroxidation of biological membranes (Sharma and Agarwal, 2004). *S. racemosa* treated groups showed alleviation of SOD and catalase enzyme activities and prevention in lipid peroxidation formation indicating free radical scavenging activity. Similarly results were seen on administration of triphala in cold restraint stress (Dhanalakshmi, et al., 2006).

Further the data was supported by histopathological studies, where the ovaries of the stressed rats showed multiple follicular cysts of varying sizes with diminished granulosa cells and some atretic follicles having degenerated oocyte with disaggregation of granulosa cells in medulla and cortex. Corpus luteum and follicles were replaced by collagenous tissue (corpus fibrosum) due to the non-availability of steroidal hormones, which are essential for their maturation and differentiation, local estrogen produced by granulosa cells, imbalanced endogenous steroid and protein hormones or due to generation of free radicals like ROS (Fig 2). Rats treated with *S. racemosa* 100mg/kg showed some of the follicles with normal follicular development with intact oocyte and corpus luteum replaced by collagenous tissues (Fig 3). At 200mg/kg b.w showed most of the follicles with normal follicular development with intact oocyte and the corpus luteum appears intact (Fig 4). The above changes may due to the increased secretion of FSH and LH at HPA, availability of steroidal hormones or reversal of stress induced oxidative stress.

CONCLUSION

Thus ethanolic extract of *Symplocos racemosa* at both doses is useful in relieving stress induced female reproductive disorders.

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Table- 1: Effect of ethanolic extract of *Symplocos racemosa* (EESR) bark on mean number of days in different phases of estrous cycle (28 Days) in rats.

Groups	Proestrous	Estrous	Metestrous	Diestrus
Vehicle control	4.16±0.16	5.80±0.20	7±0.25	11±0.44
CRS (4°C for 3h)	15.16±0.54 ^{***a}	2.5±0.22 ^{***a}	2.83±0.16 ^{***a}	7.83±0.16 ^{***a}
CRS + EESR (100mg/kg)	8.66±0.21 ^{***b}	4.33±0.21 ^{***b}	4.5±0.24 ^{***b}	10.33±0.21 ^{***b}
CRS + EESR (200mg/kg)	6.16±0.16 ^{***b}	5.16±0.16 ^{***b}	6.16±0.16 ^{***b}	10.5±0.22 ^{***b}

- Values are expressed as Mean ± SEM.
- Data were analyzed by one way ANOVA followed by Dunnett's "t" test.
- Number of animals in each group n = 6.
- ^aComparison made with vehicle control group; ^bComparison made with CRS group.
- ****P*<0.001; ***P*<0.01

Table- 2: Effect of ethanolic extract of *Symplocos racemosa* (EESR) bark on different organ weights (g/100 g of body weight) in female rats.

Groups	Ovaries	Uteri	Adrenal glands
Vehicle control	0.04±0.01	0.28±0.01	0.01±0.01
CRS (4°C for 3 h)	0.02±0.01 ^{***a}	0.05±0.01 ^{***a}	0.02±0.01 ^{***a}
CRS + EESR(100 mg/kg)	0.03±0.01 ^{***b}	0.18±0.01 ^{***b}	0.01±0.01 ^{***b}
CRS + EESR (200 mg/kg)	0.04±0.01 ^{***b}	0.20±0.01 ^{***b}	0.01±0.01 ^{***b}

- Footnotes are same as given in table-1.

Table- 3: Effect of ethanolic extract of *Symplocos racemosa* (EESR) bark on serum glucose, cholesterol and triglyceride levels (mg/dL) in female rats.

Groups	Glucose	Cholesterol	Triglycerides
Vehicle control	128.50±1.77	69.03±1.17	95.62±0.97
CRS (4°C for 3h)	76.25±0.59 ^{***a}	39.05±0.68 ^{***a}	72.07±1.10 ^{***a}
CRS + EESR(100 mg/kg)	99.08±1.23 ^{***b}	56.99±1.16 ^{***b}	77.33±1.28 ^{***b}
CRS + EESR (200 mg/kg)	102.40±1.60 ^{***b}	61.42±2.09 ^{***b}	91.43±1.65 ^{***b}

- Footnotes are same as given in table-1.

Table- 4: Effect of ethanolic extract of *Symplocos racemosa* (EESR) bark on ovary and uterus antioxidant enzymes - SOD, catalase and lipid peroxidation levels in female rats.

Groups	SOD (units/mg of protein)		Catalase (units/mg of protein)		Lipid peroxidation MDA (nano mol/mg of protein)	
	Uterus	Ovary	Uterus	Ovary	Uterus	Ovary
Vehicle control	123.69±3.88	130.47±3.88	0.36±0.01	0.27±0.01	211.36±3.50	66.70±1.65
CRS (4°C for 3h)	56.45±0.71 ^{***a}	65.11±0.89 ^{***a}	0.11±0.01 ^{***a}	0.06±0.01 ^{***a}	365.67±2.99 ^{***a}	173.40±2.08 ^{***a}
CRS + EESR (100 mg/kg)	98.02±2.90 ^{***b}	94.57±1.84 ^{***b}	0.31±0.01 ^{***b}	0.15±0.01 ^{***b}	238.99±4.49 ^{***b}	95.32±1.75 ^{***b}
CRS + EESR (200 mg/kg)	120.48±2.65 ^{***b}	121.30±1.25 ^{***b}	0.36±0.01 ^{***b}	0.19±0.01 ^{***b}	224.51±4.02 ^{***b}	74.09±0.55 ^{***b}

- Footnotes are same as given in table-1.

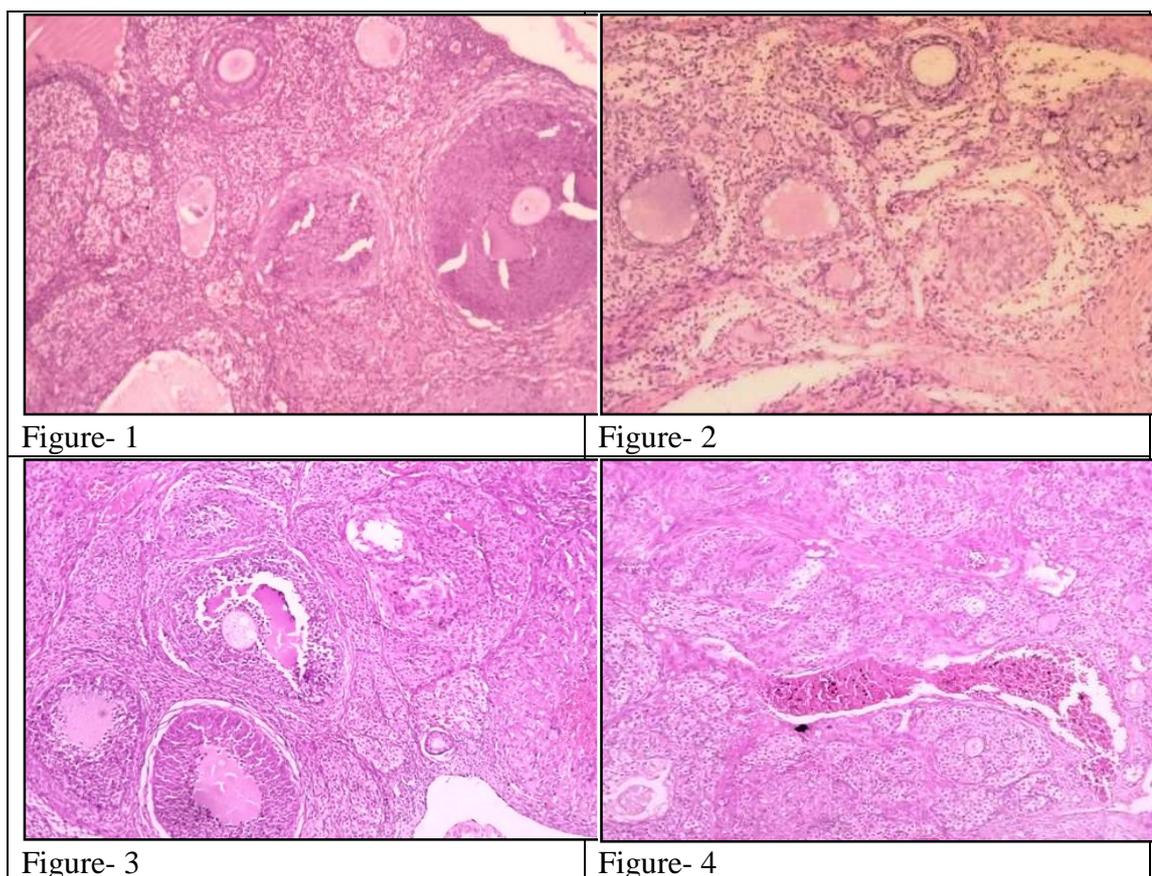


Figure-1: Section of (vehicle control) rat ovary showed normal stroma with primary and secondary developing follicles and matured graffian follicle [H and E, 100x].

Figure-2: Section of (cold restraint stress) rat ovary showed hyperchromatic nucleus, multiple follicular cysts and atretic follicles and corpus fibrosum [H and E, 100x].

Figure-3: Section of (100mg/kg) rat ovary showed only few developing follicles [H and E, 100x].

Figure-4: Section of (200mg/kg) rat ovary showed most follicles with normal follicular development and intact corpus luteum [H and E, 100x].