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PHYTOCHEMICAL SCREENING, ANTIOXIDANTS AND FERTILITY ENHANCING POTENTIAL OF ALSTONIA SCOLARIS BARKIN MALE ALBINO WISTAR RATS

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Abstract: This study was aimed to evaluate the phytochemical screening, also to establish the evidence-based fertility enhancing potential of ethanol extract of bark of *Alstoniascholaris*in male albino Wistar rats. The test extract was evaluated up to 2000 mg/kg body weight as safe for oral acute toxicity. The antioxidant potential was evaluated by DPPH radical scavenging activity which in turn connects the oxidative stress attributes tomale infertility. In accordance to determine the male fertility enhancing potentials the test sample were administered orally for 21 daysat 100, 200 mg/kg body weight.After 27 days of experiment, rats were sacrificed. In male, the sperm count, sperm morphology, live and dead sperm count were evaluated. At 200 mg/kg body weight a significant rise in sperm count, live sperm percent which also accompanied by a decrease flattened head or dead sperm percent in comparisons to normal saline treated group with a level of significance ($P< 0.05$ and $P< 0.001$). The testes histopathology was also showing seminiferous tubules lining with germs cells containing luminal spermatozoa. In female the hormonal parameters were estimated from plasma serum which shows an increment in the progesterone, LH, FSH level in animals group treated with extract 200 mg/kg body weight. The gross morphology was also found to be improved in organs associated with reproductive system.

Keywords: *Alstoniascholaris*, compounds identification,antioxidant, rat, male, fertility.

Introduction:

The use of herbs was been in practiced since the ancient times for the purpose of healthcare procurementthroughout most human races Worldwide [1].Fertility term refers to an organism for having the ability to reproduce itself whereas the inability of carry a pregnancy or to conceive is called as infertility. An estimation has reported that the prevalence of infertility is 10-15% married population across the globe [2]. Male infertility is the failure of causing pregnancy with a fertile female. This is attributed to few reproductive diseaseswhich may include;poor sperm production and count, low quality sperms with functional defects, inadequate quality of semen which measures male fecundity[3].

Many approacheshave beenmade for evaluation of fertility enhancing potential since long duration including chemical, hormonal and immunological approaches. The treatment with imported drugs and with advance technologies cannot be afforded by majority of couples having the problem infertility. Consequently,the uses of ethnomedicines remains and also will be remain for prolong as a chief sources and method towards health care ailment in most of developing countries. Moreover,many studies revealed the pro- fertility potential of various herbal drugs. However, no such investigationhas been done on*Alstoniascholaris*to the best of our knowledge.

Production of reactive oxygen species (ROS) or free radical from different environmental sources and metabolic pathwaysleading many changes in biological organ functioningwith attribution towards lipid peroxidation and

formation of many diseases **[**4**].**Recent research has suggested that the plants sources of medicine possess antioxidants potentials may correlates to theresistance against many graded diseases among these infertility also found to be implicated as oxidative stress hinders the reproductive functions.

*Alstoniascholaris*is mostly abundant medicinal plant popularly known as "the Devil tree" or "Saptaparni".It is most common tree grew up to a height of around 3.0 meterfound in India, Bangladesh,and South East Asia. This plant is available predominantly in Assam and the North-Eastern region in India. *Alstoniascholaris*has wide range of applications in treating life threatening diseases like diabetes, cancer, liver problems and even has strong antioxidant, analgesic, fertility enhancement anti-inflammatory, anti-diarrhoeal and antimicrobial activity [5].The various extracts of this plants reported with the absence or presence many chemical constituents such as alkaloids,carbohydrates, flavonoids, proteins, oils, tannins, phenolics, carbohydrates, isoflavone glycoside, and sesquiterpene lactoneswhich justify its diverse medicinal value [6].

Figure 1:*Alstonia scholaris*

MATERIALS AND METHODS

Collection and authentication of plant materials: The fresh bark of *Alstoniascholaris* were collected from the locality of BondaAmgaon of Kamrup (rural) district, Assam, India. The herbarium was prepared and authentication of plant species was done in GauhatiUniversisty, Assam, India.

Extraction: The extractionwas done by the process of cold maceration using 100 grams of shed dried powder of plant bark extracted with ethanol as a solvent. The obtained preparation was filtered and the filtrates are concentrated to sticky mass using Rota evaporator 3.42% was calculated as extractive value[7].

Pharmacognostic study: The preliminary test Phytochemical constituents such as test for alkaloids, glycosides, flavonoids, steroids, phenanthrene, phenolic compound and tannins were done [8].

Acute Toxicity Study: As per the OECD guidelines the oral acute toxicity study was carried out on the experimental rats.

***In-vitro* antioxidant activity:**

DPPH radical scavenging activity: Antioxidants potentialactivitywas measured by 2-2-diphenyl-2-picrylhydrazyl (DPPH). One ml of 0.1 m mol solution of DPPH in ethanol was mixed with 0.3 ml of ethanolic extract of*Alstoniascholaris* bark in various concentration (20, 40, 80, 160 mcg/ml) was prepared for 30 minutes at room temperature.The absorbance was recorded at 517 nm using UV visible spectrophotometer. Each sample was tested against an appropriate blank. A positive control without extract was set up in parallel. Ascorbic acid as a standardwas tested at different concentrations. The free radical scavenging was determined base on the percentage of DPPH radical scavenged using the following equation-

$$\% \text{ Inhibition} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$

Where A sample is the absorbance of a sample solution, and A control is the absorbance of the control solution (containing all of the reagents, except the test sample) [9, 10].

Experimental animals: A sum of 24 Albinowith an average weight of 190 gm-250gm of 3 months of age were acclimatized for 15 days in the animal housed facility of Assam down town University, Assam India.The animals were maintained6 animals in each metabolic cage with average temperature 28-31°C, 12 hours dark and light with proper ventilation and supply of food pellets and water. Experiments was conducted in compliance with the protocols prescribed by OECD and CPCSEA guidelines. It wasapproved by the institutional animal ethics committee

in accordance with the CPCSEA nominees with minimal animal suffering the approval no and date given asAdtU/IAEC/2022/08 on 24/09/2022. The rats are randomly divided into four different groups and the treatment was given orally for control group distilled water is administered as 1 mL/kg body weight,the ethanol extract of *Alstoniascholaris*were administered as 100 and 200 mg/kg body weight and a standard drug treated group administered with 0.3 mg/kg of clomiphene citrate. The treatment was continued for 14 days the changes in body weight and total food consumption were recorded during the experimental days. At 15th day from the starting of treatment the animals were sacrificed with adequate euthanasia, blood sample were collected for biochemical estimation of reproductive hormones and other blood counts and lipid profile. The ventral prostrate were removed cleaning the attached fats and weighed. The epididymis, seminal vesicles andtestis, were isolated, right testis were processed for histopathology whereas with epididymis and left testis are considered for sperm count and sperm morphology [11].

Gonadosomatic Index (GSI). The isolated epididymis and testes were weighed and recorded for all groups of animals and gonadosomatic index was determine using the formula as below:

{testes weight ÷ body weight} x 100 [12]**.**

Sperm analysis: The scrotal incision was made to collect epididymis which is kept in petri-dish. Epididymis is weighed and recordedfor all the groups of rats. The crushed and homogenized epididymis was transfer into another petri dish preloaded with 1 ml normal saline solution which then added to semen and mixed thoroughly with the aid of syringe to release and draw continuously to achieve adequate mixing. The mixture of semen was sucked up to 0.5 mark within the red blood cell pipette, addition of normal saline solution was done till 101 mark of the pipette and mixed, then the normal saline was discarded from stem of the pipette and the contents from bulb of the pipette was mixed thoroughly. One drop from the mixture was transferred to the neubauerchamber and spreadedthrough capillary action by placing a cover slip. The neubauer chamber was placed and viewed under 40X magnification in a slide stage microscope.The sperm cells were counted within the major five squares of neubauer chamber sliding the stage left and bottom to right and top. The counting of sperm was calculated as = n x $1x10^{-6}$ /ml of semen in rat [13].

Hormonal analysis: The collection of blood was done from tail veil of all the experimental animals after completion of the treatment. Centrifugation of blood samples was done for10 minutes at 2500 rpm. The supernatant serum was withdrawn for estimation ofreproductive hormones testosterone, luteinizing hormone (LH) andfollicle-stimulating hormone (FSH). Serum FSH and LH was determined by a radioimmunoassay (RIA) kit, Board of Radiation and Isotope Technology, Mumbai, India); FSH level was measured by a microplate chemiluminescence immunoassay (CLIA) kit [12]. The testosterone concentration of serum was estimated by an enzyme-linked immunoassay method (TransasiaBiomedicals Ltd., Bombay, India) following all the instructions and protocol designed in the commercial kit [14].

Histological analysis: The collected right testis was fixed for 24 hours in Bouinfluid then it dehydrated using 70% alcohol. The dehydrated tissue again was washed through 80%,90% and absolute alcohol followed by treatment with xylene for several periods. It was finally fixed and infiltrated in paraffin wax keeping in oven at 65ºC for 1 hour.The embedded tissue was sliced using hand rotary microtome with continuous thin sections of 5 microns. Sections of tissues were placed in slides preloaded with albumin and allowed to dry for 2minutes using a hot plate. The tissue slides then treated with xylenefor dewaxing and also treated with several alcohol concentration in descending order.Haematoxylin-eosin was used for staining the slides and finally it was mounted in DPX. It was observed under at a 100X magnification under Axio-Cam microscope [15].

Result and discussion:

Phytochemical Screening: The ethanol extract was processed for theDetection of alkaloids, glycosides, flavonoids, steroids, phenanthrene, phenolic compound and tannins obtained results shown in **Table 1.**

Figure 1: Steps involved in extraction and phytochemical tests

Table 1: Phytochemical screening of ethanol extract of *Alstoniascholaris* bark

Sl. No.	Constituents	Test	Methanolic Extract
1	Alkaloids	Mayer's reagent	++
		Dragendroff's reagent	++
		Hager's reagent	++
		Wagner's reagent	++
2	Flavonoids	Aqueous NaOH	++
3	Glycosides	Borntrager's reagent	++
		Legal test	++
		Keller kiliani test	++
4	Phenols	Ferric chloride test	++
		Lead acetate test	++
5	Carbohydrate	Molisch's reagent	++
		Fehling's reagent	++
		Benedict reagent	++
		Seliwanoff's reagent	--
6	Saponin	Foam test	++

++ (Positive tests), -- (Negitive tests)

Acute Toxicity Study: The acute oral toxicity was of the ethanol extract was evaluated up to a dose of 2000 mg/kg body weight, where no sign of toxicity and mortality was observed so, the oral LD50 thus taken to be > 2000 mg/kg.

***In-vitro* antioxidant activity study:**

In-vitro investigation of methanolic extract of *Alstoniascholaris* barkwas carried out using DPPH radical scavenging activity method with the help of a standard anti-oxidant ascorbic acid of different concentration as shown in the table below (Table-2).

Table 2: Free radical scavenging activity (DPPH) of ethanolic extracts of *Alstoniascholaris* bark

Test items	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50} (µg/ml)
ethanolic Extract	10	0.0715	32.06	38.31
	20	0.0632	39.25	
	30	0.0614	42.36	
	40	0.0502	51.36	
	50	0.0493	58.29	
Ascorbic acid	10	0.0864	34.05	34.81
	20	0.0774	41.69	
	30	0.0698	43.36	
	40	0.0666	53.69	
	50	0.0616	61.25	

The percentage of inhibition obtained were ranging from 32.06 at 10µg/ml to 58.29 at 50 µg/ml with 38.31 as anIC_{50} (µg/ml) for the extract and for positive control (ascorbic acid) were 34.05 at 10µg/ml and 61.25 at 50 µg/ml with 34.81 as anIC_{50} (µg/ml). (**Table: 2**). The obtained data suggestthe ethanolic extracts of *Alstoniascholaris* bark exhibits hydrogen donating capabilities and resulting the scavenging of free radicals.

Gonadosomatic Index: Effect of repeated daily oral treatments with 100-200 mg/kg/day of ethanol extract of *Alstoniascholaris*(EEAS) on the average body weight on initial day and one day after the termination of experiments, testicular weight (TW) and gonadosomatic indices (GSI) and represented with a significant increase at $p<0.05$, $p<0.01$, and $P<0.001$ respectively when compared with the normal saline treated groups
as shown in the **table 3** below.

Table 3: Effect ofethanol extract of *Alstoniascholaris*(EEAS) on rats GSI, TW

Treatment	Average body weight on Day-1(gm)	Average body weight on Day-15(gm)	Average testicular weight (gm)	Average gonadosomatic index ($x10^{-2}$)
Control	225.40±12.16	226.20±13.16	03.46 ± 0.32	12.04 ± 0.16
100 mg/kg	227.80±12.95	228.40±12.95	03.50 ± 0.16	12.80 ± 0.42
200 mg/kg	230.40±13.37	232.80±13.16*	03.84 ± 0.43*	13.08 ± 0.81*
Clomiphene citrate 0.3 mg/kg	228.70±12.65	230.70±13.95	04.25 ±0.32	13.60 ± 0.32

* Represent significant increases at $p<0.05$, $p<0.01$, and $P<0.001$ respectively when compared with the normal saline treated groups

Sperm analysis: The average sperm measuredin the EEAS treated groups was found to increase as compared to the control group with a level of significantly ($P < 0.05$) however it was less than the standard drug clomiphene citrate treated group. Range of normal sperm and abnormal sperm percentage was found to be improved in comparison to the normal saline treated groups as shown below **Table 5**.

Table 5: Effect of ethanol extract of *Alstoniascholaris*(EEAS) on sperm parameters

Parameters	Control (Saline)	100 mg/kg EEEF	200 mg/kg EEEF	0.3 mg/kg Clomiphene citrate
Sperm count ($x10^{-6}$/ml)	78.72 ± 0.16	80.54 ± 0.16	83.20 ± 0.01	85.66 ± 0.20
Abnormal sperm (%)	13.26 ± 1.04	12.85 ± 0.40	11.86 ± 0.32	11.05 ± 0.4
Normal sperm (%)	85.84 ± 0.32	86.15 ± 1.20	87.08 ± 1.02	88.95 ± 0.16

Values are expressed as Mean ± S.E.M; n = 6 in each group

Hormonal analysis: TheSerum levels of follicle stimulating hormone, luteinizing hormone and testosterone were measured using commercially available kits. According to the **Table 6,** the groups of animal with 100 mg/kg body weight and 200 mg/kg body weight of ethanol extract of *Alstoniascholaris*treated were obtained with a certain rise in the hormonal concentration with a level of significance ($P < 0.01$) and($P < 0.001$) respectively, whereas it was less when compared to the standard drug clomiphene citrate treated group as shown below.

Table 6: Effect of ethanol extract of *Alstoniascholaris*(EEAS)on testosterone level, FSH and LH

Parameters	Control (Saline)	100 mg/kg EEEF	200 mg/kg EEEF	0.3 mg/kg Clomiphene citrate
Testosterone (ng/ml)	3.95 ± 0.27	4.10 ± 0.16	4.68 ± 0.20	4.95 ± 0.16
FSH (mlu/ml)	3.40 ± 0.20	3.85 ± 0.40	3.95 ± 0.18	4.14 ± 0.4
LH (mlu/ml)	30.16 ± 3.18	32.46 ± 1.40	34.12 ± 0.60	36.20 ± 0.12

Values are expressed as Mean ± S.E.M; n = 6 in each group

Histopathology: The testicular sections of control group (**a**), 200 mg/kg ethanol extract of *Alstoniascholaris*(**b**)and a standard drug clomiphene citrate treated group (**c**) photographs are being presented below in **Table 5.** In (**b**) the observation of spermatogenic series (SS) in seminiferous tublules, spermatozoa containing lumen (L),spermatogonium (SG), arrow marked represented spermatids and spermatozoa were improved compared to the normal saline treated group (**a**). However, the improvement was low in reference to the standard drugs treated group (**c**).

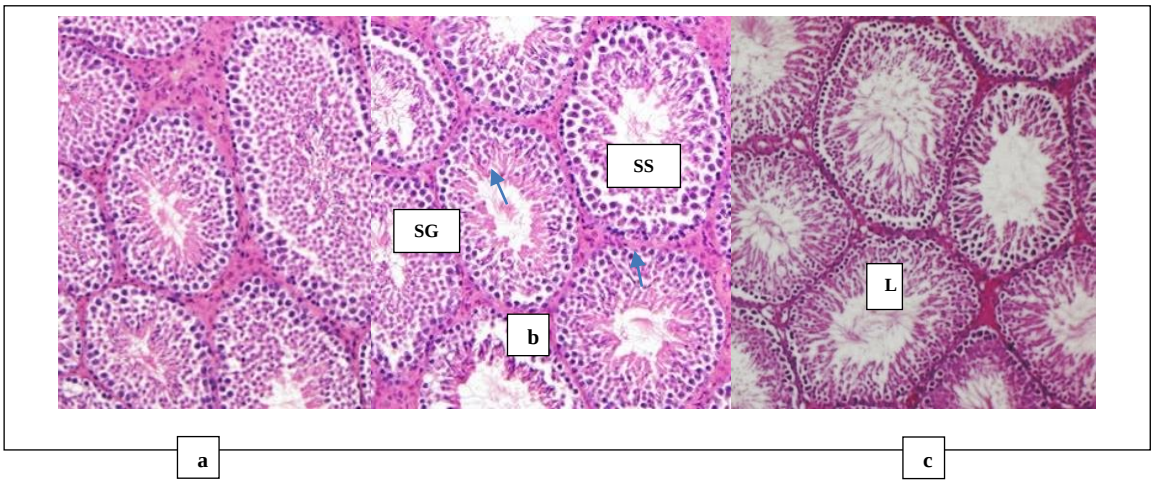

Figure 5: Histopathological Photomicrographs of rat testis

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