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IN VIVO ANTIOXIDANT, ANTI-INFLAMMATORY AND TLC PROFILING OF INDIAN KINO IN RAT MODEL

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ABSTRACT

Background: Polycystic ovary syndrome (PCOS), the most common endocrine and metabolic disorders of reproductive-aged women, is characterized by menstrual disturbance, infertility, oxidative stress and ovary inflammation. Herbal medicines are known to cure these disorders with fewer side effects. Among them, *Pterocarpus marsupium*, which is commonly known as Indian Kino, has a long history of numerous traditional and ethnobotanical applications in diverse cultures. **Objective:** To investigate invivo antioxidant and anti-inflammatory activities of *P.marsupium* along with TLC profiling. **Methodology:** *In vivo* antioxidant activity was carried out in PCOS induced female albino rats as described by *Mamata et.al* (2013). *In vivo* anti-inflammatory activity was measured by carrageenan induced hind paw oedema assay. Thin layer chromatographic profiling of methanol extract was also conducted to identify the bioactive compounds. **Results:** Upon administration of low and high dose of *Pterocarpus marsupium* methanol extract to PCOS induced rats indicated increase in CAT, GSH and SOD enzymes but decreased in MDA tissue content when compared to normal control and PCOS treated group. The extract has also exhibited dose dependent anti-inflammatory activity. TLC profiling of methanol extract confirms the presence of various phytochemicals. **Conclusion:** The results of methanol extract of *P.marsupium* indicates significant *in vivo* antioxidant and anti-inflammatory activities, which can be considered as a promising plant source of natural antioxidant and anti-inflammation that is due to the presence of phytoactive compounds confirmed through TLC.

KEYWORDS: *Pterocarpus marsupium*, SOD, CAT, carrageenan hind paw oedema, female albino rats, TLC.

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women, with prevalence rate ranging from 5% - 21%.[1,2] It is characterized by elevated luteinizing hormone, chronic anovulation with oligoamenorrhea, enlarged cystic ovaries, obesity, hyperandrogenism and infertility. PCOS patients usually have a high oxidative profile which causes disturbance in antioxidants balance, leading to harmful effects of reactive oxygen species (ROS) including infertility, endometriosis, injury to ovarian epithelium's DNA, excessive apoptosis and alteration in cell signaling process.[3,4] In addition, the resultant oxidative stress induces an inflammatory environment furthering elevated insulin resistance, contributing hyperandrogenism, dyslipidemia and hypertension.[5] Regardless of abundant severe investigations that were carried out on PCOS, a distinct single etiology of this disease remains unclear. Antioxidants act as free-radical scavengers by safeguarding living entity from destruction generated by abnormal ROS production.[6] However, conventional synthetic drugs cause many serious side effects such as risk of ovarian hyperstimulation, multiple pregnancies as well as psychological effects.[7] This has enabled to search for natural antioxidants which are therapeutically effective with minimal side effects, safety, efficacy and acceptability as encouraged by World Health Organization. People from different areas have used particular parts of medical plants to treat the health problems.[8]

Pterocarpus marsupium Roxb. is commonly called as Indian Kino (English), Bijasal (Hindi) and Raktahonne (Kannada). It is native to India, Nepal and Sri Lanka, belonging to family Fabaceae.[9] It grows medium to large sized tree reaching height up to 15-20 meter with dark brown to grey bark having swallow cracks, which exudes a red gummy substance called 'Gum Kino' when injured. Bark of this plant is considered as good source of natural antioxidants for free radical mediated ailments.[10] A variety of flavonoids and their derivatives

have been isolated from various parts of the plant. The wood and bark of the tree is useful for diabetic patients.[11] The heart wood, leaves, flowers and bark have useful medicinal properties. Based on literature survey, our work is the first report on the effects of *P.marsupium* as antioxidant and anti-inflammatory agent in PCOS induced rat. Thus the ultimate goal of the current study is to test out the efficacy of *P.marsupium* for the treatment of antioxidant and anti-inflammatory activities in rat model.

MATERIALS AND METHODS

Plant material

Pterocarpus marsupium Roxb. bark was collected from Kalaburagi district, Karnataka, during the month of June, 2015. The plant was authenticated by Dr. Imran Baig, Professor, Botanical garden, UAS, GKVK Campus, Bangalore and the voucher specimen (UASB:4552) was deposited in the herbarium of the GKVK botanical garden.

Freshly collected bark of *Pterocarpus marsupium* Roxb. was washed thoroughly, shade dried and then powdered to required particle size. The bark powder (100g) was successively extracted by Hot Soxhlet extraction with polar solvent methanol. The extract was heated at 30-40ºC in hot air oven till the solvent got evaporated. Dried extract was kept in refrigerator at 4ºC for future use.

Experimental animals

Rats were (weighing 180–200g) obtained from NIN, Hyderabad, housed in polypropylene cages and maintained at 25°C; 45% humidity in 12hr light/dark cycle. They were fed on pellet diet (Amrut laboratory animal feed diet Pune, Maharashtra, India) and water *ad libitum*. All experimental procedures were carried out in strict accordance with the guidelines prescribed by the committee for the purpose of control and supervision on experimentation on animals (CPCSEA Reg. No-34800/2001) and were approved by the Institutional Animal Ethical Committee.

Acute toxicity studies

The acute toxicity study was performed on adult rats dividing all the rats into four groups containing minimum six animals in each group.[12] They were fasted for 18 hr with water ad libitum. The methanol extract suspension was prepared and administered orally at three different doses of 250,500 and 1000 mg/kg body weight respectively to different groups of rats separately. Control rats received the vehicle (Tween-80, 1%, p o.) only. The animals are kept under observation for 72 hours for behavioral changes and mortality check up.

Antioxidant activity

Experimental Design

All the experimental animals except control group were injected with Testosterone Propionate (TP) intraperitoneally at the dose of 1mg/100gm b.w dissolved in olive oil once daily for 15 days. Control group received vehicle (olive oil) only. Vaginal smears from all experimental animals were collected daily and evaluated microscopically to confirm the induction of PCOS. PCOS induced positive animals were randomly divided into four groups of minimum 6 rats per group as described by Mamata et.al, with slight modifications[13], which includes a control group that received vehicle only (1% aqueous solution of carboxymethyl cellulose) once daily *p.o*. The first PCOS induced group was left for natural recovery for 15 days post TP treatment. The second PCOS group was given repetitive dose of 10mg/kg Clomiphene citrate (Positive control). The other two groups were given repetitive doses of 200mg/kg (low dose) and 400mg/kg (high dose) of methanol extract of *P.marsupium* bark once daily *p.o* for 15 days post TP treatment. At 24 hr after the last dose of treatment and 18hr fasting period, the rats were anaesthetized with diethyl ether. The ovaries were dissected for evaluation of antioxidant activity.

Preparation of ovary homogenates

200mg of dissected ovary tissue was homogenized in 10 volumes of 100 mmol/L KH_2PO_4 buffer containing 1mM EDTA, pH 7.4 and centrifuged at 3500g for 4 minutes at 4°C.[14] The supernatant obtained was used for determination of different antioxidant enzyme activity.

Catalase (CAT)

50 µL of the lysate was added to a cuvette containing 2 mL of 50mM phosphate buffer (pH 7.0). The reaction was initiated by adding 1mL of fresh 30mM hydrogen peroxide and the decomposition rate of hydrogen peroxide was measured at 240nm for 5min on a spectrophotometer.[15] A molar extinction coefficient of 0.041mM−1 cm−1 was used to calculate catalase activity. One unit of activity is equal to 1mmol of H_2O_2 degraded per minute and expressed as units per milligram of protein.

Superoxide dismutase (SOD)

100µL of each homogenate of different experimental groups as well as positive and negative control was added to tubes containing 0.5mL of carbonate buffer and 0.5 mL of EDTA solution.[16] The final volume was made up to 2.5mL.The reaction was initiated by the addition of 0.5 mL epinephrine and increase in absorbance at 480 nm was measured in a Systronics 119 UV spectrophotometer. One hundred percent auto oxidation of epinephrine to adrenochrome was performed in a control tube without the enzyme. The enzyme unit of activity was defined, as the enzyme required for 50% inhibition of epinephrine autooxidation.

Determination of non-enzymatic antioxidant status GSH assay

10% TCA was added to each homogenate and centrifuged. 1mL supernatant was treated with 0.5mL Ellman's reagent (19.8 mg of dithiobisnitrobenzoic acid in 100 mL of 0.1% sodium nitrate) and 3.0 mL of 0.2 mol/L phosphate buffer (pH 8.0, 6.8 g of KH_2PO_4 and

17.9% of $Na_2HPO_4.12H_2O$ dissolved in 500 mL distilled water).[17] The absorbance was read at 412 nm and the GSH content was calculated according to the following calculation.

$$C_O = \frac{OD@412 \times D}{e}$$

Where, C_O is concentration of the GSH, e is extinction coefficient and D is dilution factor.

Estimation of lipid peroxidation status (TBARS)-MDA assay

100 µL of each tissue homogenate was treated with 2mL mixture [1:1:1 TBA-TCA-HCl reagent) (thiobarbituric acid 0.37%), 0.25N HCl, 10% TCA] and incubated for 45min in a boiling water bath (100°C), cooled and centrifuged at room temperature for 10min at 1000 rpm.[18] The absorbance of clear supernatant was measured against reference blank at 535 nm. TBARS concentrations were calculated using the MDA extinction co-efficient 1.56 $x10^5M^{-1}$ cm.

Ovarian histomorphology

Excised ovaries was fixed in 10% formalin and subjected to tissue processing by dehydration through an ascending ethanol series, clearing in xylene and embedding completely in paraffin wax into blocks. The blocks were then serially sectioned at 5µm thickness using microtome and were mounted on poly-lysine coated slides, deparaffinised using xylene, rehydrated and stained with hematoxylin-eosin and dehydrated, cleared and mounted on DPX and placed glass cover slips. The slides were then observed under light microscope connected to a camera to capture images.

Anti-inflammatory activity

Anti-inflammatory activity was measured using carrageenan induced hind paw oedema assay.[19] Wistar albino rats were divided into four groups of six animals in each group. The group I received normal saline, treated as control and group II received standard drug (Diclofenac sodium 10 mg/kg, *p.o.*). The test groups III & IV have received low dose 200 and high dose 400 mg/kg, *p.o.* of *Pterocarpus marsupium* methanol extract respectively. The methanol extract was suspended in 1% tween 80 and administered orally to rats one hour before carrageenan injection. One hour after administration, acute paw oedema was induced by injecting 0.1 ml of 1% (w/v) carrageenan solution, prepared in normal saline into the subplantar tissue of the right hind paw of rats. Increasing of carrageenan induced inflammatory paw volume was measured at 0, 1, 2, 3, 6, 12 hours by using vernier calipers. The anti-inflammatory activity was calculated by using the relation % inhibition of oedema = $T - T_0 / T \times 100$.

T-Thickness of paw in control group; T_0- Thickness of paw oedema in the treated group.

TLC profile

TLC plates (Merck, 20 × 20 cm) are thickly coated with silica gel "G", activated at 100 °C for 30 minutes was used for preparative thin layer chromatography (PTLC). The methanol extract was applied on plate and developed in n-butanol-acetic acid-water solvent system (6.5:1.5:2.5). The chromatogram was air-dried. Later, anisaldehyde sulfuric acid was sprayed on the surface of the plate, incubated for 20 minutes at 100°C, visualized under visible light and also in iodine chamber. The spots were marked and the R_f values were calculated.[20]

Statistical analysis

Data was expressed as mean ± standard error of mean (SEM). The collected data was subjected to One-way ANOVA (Analysis of Variance) test. The analysis was carried with Graph pad prism 6.0 software. Statistical difference was considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Acute toxicity studies

No mortality and changes in the behaviour was observed in all the treated and control groups of rats up to a dose of 1000 mg/kg body weight. Hence, 200 and 400 mg/kg body weight drug was used as low dose and high dose for invivo studies. However, methanol extract of *Pterocarpus marsupium* was administered orally for 15 days, no mortality and changes in the behaviour were observed in all groups which signify no sign of chronic toxicity.

Antioxidant activity

Table 1: Effect of methanol extract of *Pterocarpus marsupium* on antioxidant enzymes in PCOS induced rat ovary.

Groups	CAT (U/mg)	SOD (U/mg)	GSH (µm/mg)	MDA (TBARS) (nM /g)
Normal Control	50.16±0.342	12.09±0.141	65.25±1.650	15.85±0.258
PCOS Control	24.78±0.741	4.06± 0.524	30.02±1.195	29.47±0.335
PCOS Recovery	25.58± 0.882	4.12±0.553	31.35±1.111	30.52±0.284
PCOS+Clomiphene citrate	47.72±1.215	11.91±0.186	62.87±1.081	18.26±0.185
PCOS+ PM 200mg/kg	39.55±1.151*	7.33±0.253*	56.21±1.026*	22.45±0.119*
PCOS+PM 400mg/kg	45.23±0.456*	9.87±0.113*	60.25±0.985*	16.95±0.101*

* $p < 0.05$-significant compared to control group.

In vivo antioxidant assays are usually important to confirm the *in vitro* activity of certain antioxidants.[21] To assess this in animal models, a state of oxidative stress needs to be induced and treated with plant extract for observation of ability of the plant extract to scavenge free radicals and prevent organ damage. The *in vivo* antioxidant activities of *Pterocarpus marsupium* for methanol extract at a dose of 200 mg/kg b/w and 400 mg/kg b/w on PCOS induced rat ovary was illustrated in Table-1. In the present study, it was proved that the intoxication of PCOS induced in rat has caused a disturbance in the antioxidant defense system, inducing oxidative stress, leading to decrease in the CAT, SOD and GSH activities where as MDA level was significantly increased ($p < 0.05$). The CAT activity of control rat was found to be 50.16±0.342 (U/mg), whereas in PCOS treated animals it was found to be 24.78±0.741 (U/mg). Upon administration of low and high dose of *Pterocarpus marsupium* methanol extract in PCOS induced ovary has increased the CAT activity as 39.55±1.151 and 45.23±0.456 respectively. Similarly, SOD and GSH activity were also significantly diminished in the PCOS group and concomitant treatment with *P.marsupium* has restored their activities. A significant decrease in MDA activity in ovary tissues was determined when compared to PCOS treated group. This is an unison observation with the earlier reported antioxidant activity of Curcumin.[22]

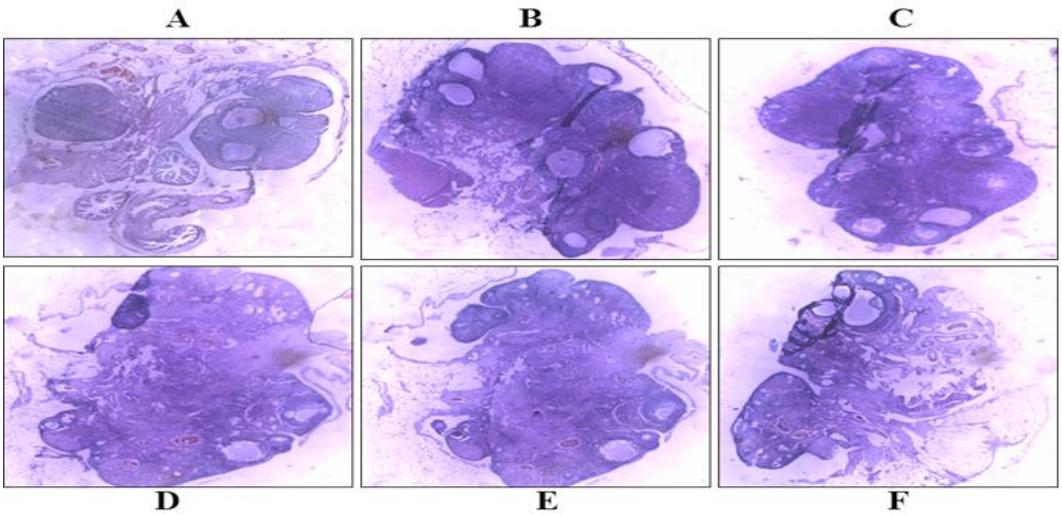


Figure 1: A: Normal control ovary, B: PCOS induced ovary, C: PCOS ovary left for natural recovery, D: PCOS ovary with Standard clomiphene citrate, E&F: PCOS ovaries with *P.marsupium* methanol extract 200mg/kg and 400mg/kg b.w respectively.

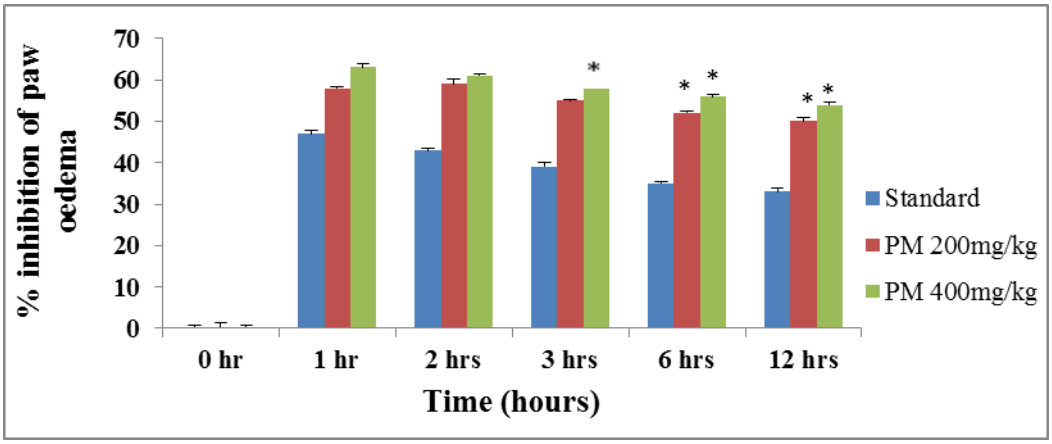
Oxidative stress is considered as an important pathological feature of PCOS and PCOS women have decreased total antioxidant status.[23] Sections of ovaries from control group animals showed healthy follicles and normal corpus luteum formation.TP induced PCOS rats exhibited numerous cysts with atretic ovarian follicle and absence of corpus luteum indicating anovulation. When rats with PCOS were left for natural recovery, the recovery was not to its potential as compared to plant drug and positive control clomiphene citrate treatment as there were few cysts and atretic follicles were still found in the ovary. However, clomiphene citrate alone treatment has led to minimum disappearance of cysts, appearance of healthy follicles and corpora lutea. Sections from low dose (200mg/kg) of *P.marsupium* methanol extract treatment exhibited normal sized healthy follicles and few corpora lutea. Cysts were dissolved, remain absent and large sized follicles were found in sections of ovary from high dose (400mg/kg) treatment group as shown in Figure-1**.** Similar results were observed in PCOS induced rats treated with *Soy isoflavones* treatment which has caused the disappearance of cysts.[24]

Anti-inflammatory activity

Table-2: Effect of *P.marsupium* methanol extract on carrageenan induced rat paw oedema volume.

Groups	0hr	1hr	2hrs	3hrs	6hrs	12hrs
Negative Control	0.31±0.02	0.31±0.02	0.31±0.02	0.31±0.02	0.31±0.02	0.31±0.02
Positive Control	0.78±0.04	0.91±0.08	0.86±0.05	0.75±0.04	0.71±0.03	0.65±0.01
Standard	0.5±0.05	0.47±0.03	0.43±0.05	0.39±0.01	0.35±0.02	0.33±0.04
PM 200mg/kg	0.6±0.03	0.58±0.02*	0.59±0.05*	0.55±0.04*	0.52±0.01*	0.50±0.03*
PM 400mg/kg	0.65±0.02	0.63±0.05	0.61±0.02	0.58±0.03*	0.56±004*	0.54±0.01*

* $p < 0.05$-significant compared to control group.



* $p < 0.05$-significant compared to control group.

Figure-2: Percentage inhibition of paw oedema by methanol extract of *P.marsupium*.

Carrageenan induced rat paw edema is a suitable experimental animal model for evaluating the anti-edematous effects of natural products.[25] The *P.marsupium* methanol extract was evaluated for *in vivo* anti-inflammatory activity in the carrageenan induced paw oedema in rat model and the result revealed that the extract exhibited dose dependent anti-inflammatory activity as summarized in Table-2.The methanol extract at the dose level 200 mg/kg bw and 400 mg/kg bw significantly inhibited ($p < 0.05$) paw oedema induced by carrageenan at different time intervals with standard Diclofenac sodium as reference as shown in Figure-2. Positive control refers to carrageenan induced rat without any treatment, which showed very little recovery.

Histopathological examination of liver was performed in carrageenan induced rat model. The normal control and extract treated groups showed normal cell architecture, where as standard group (Diclofenac sodium) treated rats showed mild inflammation around central vein as represented in Figure-3. This overall result coincides with the earlier findings executed by Tukappa et al.[26]

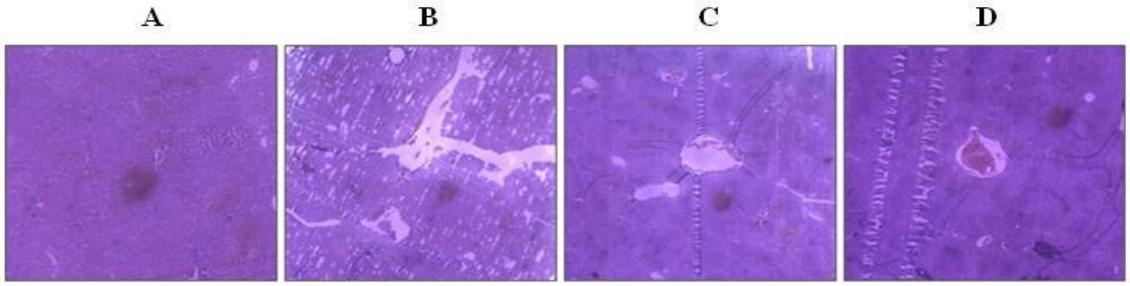


Figure-3: Histopathological observation of liver. A-Control group, B-Diclofenac sodium, C- 200 mg/kg methanol extract, D-400 mg/kg of methanol extract.

TLC profile

Thin layer chromatography was performed to generate a chromatographic profile of the extract. Based on the colour, the secondary metabolites were differentiated and R_f values were calculated as shown in Figure-4 and Table-3. This TLC profiling of methanol extract confirms the presence of diverse potent biomolecules in the plant which substantiates the work on phytochemical analysis of this extract carried out by Londonkar et al.[27]

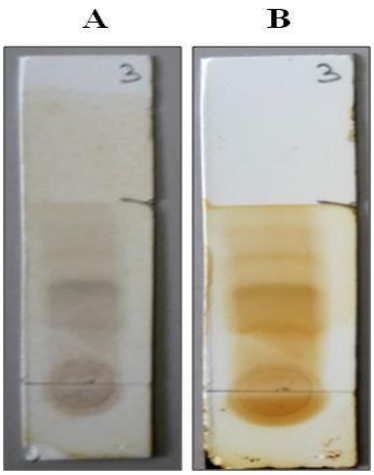


Figure 4: TLC Chromatogram of bioactive compounds from *P.marsupium* methanol extract. A-Visible light, B-Iodine chamber.

Table 3: Determination of R_f values through TLC.

Extract	Solvent System	Revealing reagent	No. of spots	R_f Value
Methanol	n-butanol: acetic acid: water	anisaldehyde sulfuric acid, iodine chamber	4	0.34, 0.6, 0.72,0.80

CONCLUSION

From the present investigation, it can be concluded that *P.marsupium* contain pharmacologically active substances such as flavonoids, phenolics, terpenoids, glycosides and saponins confirmed through TLC which are responsible for antioxidant and anti-inflammatory activities. These findings suggest that the traditional use of this plant is justified. Further, isolation and identification of bioactive constituents present in the methanol extract is currently under investigation in our laboratory.

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