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Effect of aqueous extract of *Ficus platyphylla* on female Wistar rats with estradiol valerate-induced polycystic ovarian syndrome

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Abstract

The aim of the study was to investigate the effects of *Ficus platyphylla* on female Wistar rats with estradiol valerate (EV)-induced polycystic ovarian syndrome (PCOS).

PCOS was induced in female Wistar rats with regular 4-5 day oestrus cycles via the intramuscular injection of 4 mg EV per rat in an oily solution. The rats were allowed 30 days to establish PCOS. Then, the animals were divided into five treatment groups. The positive control group received clomiphene citrate, the negative control group received distilled water and the other groups received 100, 200 and 400 mg/kg of the aqueous extract. All groups were dosed for 15 days, except the positive control group, which was dosed for 5 days. On the 16th day, the animals were sacrificed. Hormonal assays and histological studies were then conducted.

An elevated Luteinizing hormone (LH)-to- Follicle stimulating hormone (FSH) ratio is typical in many women with PCOS. The extract-treated groups showed a lower LH/FSH ratio compared with both the positive and the negative control groups. The progesterone levels were higher in the extract-treated groups compared with the negative control group, indicating luteal phase repair. The influence of the extract on ovarian morphology in the EV-induced PCOS model showed a marked reversal of polycystic ovary. These data help validate the use of this extract in folk medicine for the treatment of infertility. Considering its apparent safety, this study paves the way for an efficacy study for its use in treatment of PCOS in humans.

Keywords: *Ficus platyphylla*, polycystic ovarian syndrome, infertility, estradiol, Wistar rats.

Introduction

Plants have been used for many years to alleviate human fertility health issues. The fertility promoting activities of plant extracts, including *Lepidium meyenii* [1], *Ageratum conyzoides* [2], *Coccinia cordifolia* [3], *Kaempferia parviflora* [4] and *Ficus platyphylla* [5], have been reported. However, many of these plants have not been scientifically shown to have activity against some of the known female fertility challenges such as polycystic ovarian syndrome, endometriosis and anovulation.

Polycystic ovary syndrome (PCOS) is a disorder that is frequently characterised by ovulatory dysfunction, abdominal obesity, hyperandrogenism and metabolic disorders [6-8]. It results from abnormal (Gonadotropin releasing hormone (GnRH) pulses that give rise to elevated LH/FSH ratios, which are typical in many women with PCOS [9]. The estimated prevalence of PCOS ranges from 6 to 8 % in women of the reproductive age. However, approximately 40% of women with PCOS are affected by infertility [10, 11].

Experimentally, one of the methods to induce PCOS in rats involves the intramuscular injection of estradiol valerate (EV) [12]. The morphological changes observed are follicular cysts, with a well-developed theca cell layer and atretic follicles [12].

Various treatment modalities have been employed to manage PCOS. Clomiphene citrate (CC) is a triphenylethylene derivative that is commonly used to induce ovulation in women [13]. It is the drug of choice in managing this condition [14-16]. However, therapeutic approaches to PCOS remain an ongoing source of debate. Despite the effectiveness of the available drugs, screening plants for use in the development of new medicines that have better activity and fewer side effects is of paramount importance.

F. platyphylla Del. Holl (Moraceae), which is commonly referred to as the gutta percha tree, is a large deciduous savannah tree. It is widely distributed throughout the savannah region along the West African coast. In Northern Nigeria, where it is locally known as

"gamji" among the Hausas, the plant is used to treat several diseases, such as insomnia, psychosis, depression, and as an analgesic [17, 18]. Among the people of the Sokoto state, the decoction from this plant is traditionally used as a medicine to promote fertility.
activities [19, 20] and fertility-promoting properties [5] in rats. Additionally, a preliminary phytochemical analysis revealed the presence of flavonoids, tannins and saponins, glycosides, volatile oils and steroids in the plant [21]. Furthermore, this plant has been shown to also possess some central nervous system activity [17]. Therefore, the aim of this study is to investigate the effects of the aqueous extract of *F. platyphylla* on female Wistar rats with PCOS induced by intramuscular injection of estradiol valerate (EV).

Methods

Ethical approval

The study was approved by the Animal Research Ethical Committee, Usmanu Danfodiyo University, Sokoto (211706005) and was conducted in accordance with the Animal Research Regulation 1985- 2010 and the Organization for Economic Development (OECD) guidelines on good laboratory practice [22].

Preparation of plant material and extraction

The plants were obtained from the surroundings of the Usmanu Danfodiyo University teaching hospital Sokoto in the month of November. The plants were identified at the Taxonomy unit of the Department of Botany, Usmanu Danfodiyo University, Sokoto. A voucher specimen was also deposited in the herbarium, with the voucher accession number 003.
The leaves, stem bark and seeds of *Ficus platyphylla* were washed with tap water, cut into pieces and air dried to a constant weight. The dried materials were pulverised mechanically into a dry powder using a grinding machine. The powder was subjected to Soxhlet extraction using distilled water. The filtrates were evaporated in the oven at 45 C. The final product was a dry powder of 4.74% w/w.

Standard drugs

Clomiphene citrate (CC, branded Clomid^{R}) was obtained from Sanofiaventis (One Onslow Street, Guildford, Surrey GU1 4YS, UK). Estradiol valerate USP injection used was Progynon Depot^{R}, manufactured by Medipharm (Pvt.) Ltd., 108-Kotlakhpat Industrial Estate, Lahore.

Experimental animals

Six- to eight-week-old female virgin Wistar rats were obtained from the Veterinary institute Vom, Jos and kept in the animal house in the Department of Pharmacology, Usmanu Danfodiyo University, Sokoto. The animals were kept in well-constructed cages that allowed freedom of movement for 2 weeks and thus acclimation to the laboratory conditions before the commencement of the study. Water and standard rat chow were provided *ad libitum* throughout the acclimation period of the study. The housing conditions were maintained at 25 ± 2 C at 12 h day/night cycles.

Induction of PCOS in the animals

Sixty adult virgin Wistar rats of approximately 10-12 weeks of age, weighing between 160-180 g and with regular 4-5 day oestrus cycles as assessed by vaginal smear, were used for the study [23]. Eight of the rats were kept as controls, and the others were each given intramuscular injection of 4 mg EV in an oily solution per rat [10, 12]. Vaginal smears were examined daily in all animals. Cessation of cyclicity, which was shown by the persistent cornification of vaginal smears, was used as a criterion for selection into the PCO group. This was observed in all but 3 of the rats injected with EV by day 30. Those 3 rats were thus not used in the study.
Six animals from each group (Control and PCO) were randomly selected and anaesthetised with chloroform. Blood samples were collected by cardiac puncture, and the sera were used for serum hormonal assays (FSH, LH, estradiol, progesterone and testosterone). The ovaries were excised and weighed, and histopathological examination was conducted on the ovaries. This served as the baseline result for the study.

Study Protocol

Thirty animals in the PCO group were randomly selected and divided into five groups (n=6) and housed as such (6 rats per cage). The positive control group received 71.43 mg/kg of Clomiphene citrate, which represents the estimated rat equivalent of the human dose using the scaling factor for dose equivalence between species [24]. This dose is also consistent with the reversed scaling factor from the FDA for rat to human. The negative control group received 5 ml/kg of distilled water. The other groups received 100, 200 or 400 mg/kg of the extract. All of the groups were dosed orally by gavage for 15 days, except the positive control group, which was dosed for 5 days (the initial course of treatment with CC in humans).
On the 16th day, the animals were anaesthetised, and blood samples were collected as stated above. The serum hormonal assays and histopathological studies were carried out in the same way as described above.

Serum hormonal assays

Serum testosterone, follicle stimulating hormone (FSH), luteinising hormone (LH), progesterone and estradiol were measured using an enzyme immunoassay kit for the quantitative determination of the corresponding hormones. All kits were commercially obtained from Syntron Bioresearch, Inc. (microwell EIA; Loker Avenue West, CA, USA).

Organ weights

On the 16th day, some body organs of rats in all treatment groups except the CC group were excised and weighed; organs from rats in the CC group were instead excised and weighed on the 6th day.

Histopathological examination

The excised ovaries were fixed in Bouin's solution. They were dehydrated in an ascending series of alcohol, cleared in xylene and embedded in paraffin wax that melted at 60 C. Serial sections were mounted on 3-aminopropyl triethsilane-coated slides and dried for 24 hours at 37 C [25]. The sections on the slides were deparaffinised, hydrated and stained with Mayer's hematoxylin and eosin dyes; they were then dried and mounted for histology. The ovaries were viewed at 40x magnification using the Scope photo 3.0 imaging device (Scope Tek DCM 200 (USB 2.0, Hangzhou Scope tek Opto-Electric Co Ltd). The diameter and thickness of the cystic follicles were measured. The cystic follicles were defined by thickened and fibrotic cortex with a prominent outer theca and internal layer.

Statistical analysis

The data were analysed using the one-way analysis of variance and Student's T-test. Statistical evaluations were performed using Graph pad prism version 6, and differences were considered statistically significant at $p<0.05$

Results

Effects of EV on hormonal assays, ovarian weight and morphology at Day 30

The EV treatment resulted in a significant increase ($p<0.05$) in both the LH and FSH levels in the animals at day 30. The LH /FSH ratio was significantly different from the control group (Fig 1). The progesterone and estradiol levels in the EV treated group were significantly lower than were those in the control group ($p<0.05$). Although the testosterone levels in the EV-treated groups were higher than those of the control group, the differences were not statistically significant (Figure 1).

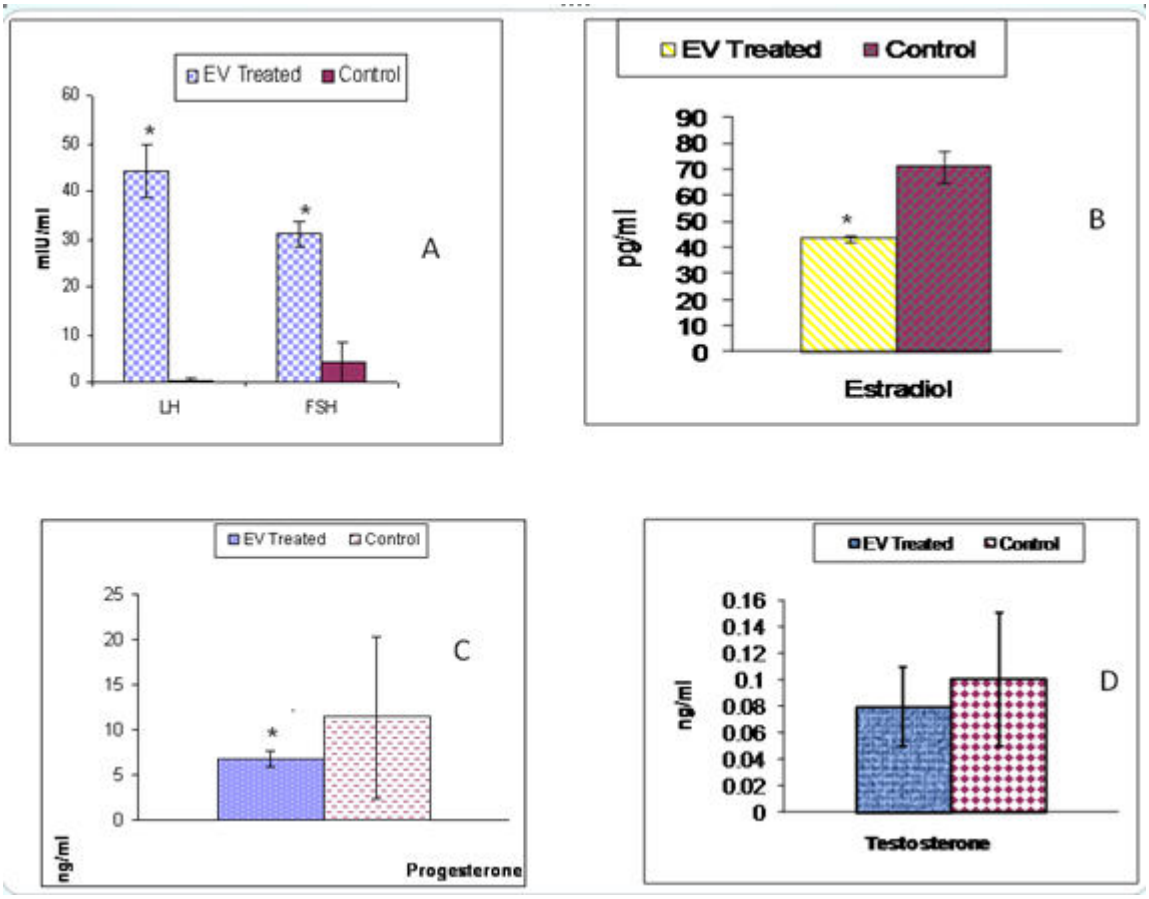

Figure 1- Effects on hormonal Assays on day 30 of EV treatment.

Results of hormonal assays on day 30 of EV treatment. LH-luteinising hormone (mIU/ml) A, FSH-follicular stimulating hormone (mIU/ml) A; E-Estradiol (pg/ml) B; PRGS- Progesterone (ng/ml) C; TST-Testosterone (ng/ml) D. Values are expressed as the mean ± SD. * Value significant at $p<0.05$, n=6.

The decrease in ovarian weight (mg) in the EV-treated rats (Figure 2) was significant ($p<0.05$). The numbers of cystic and atretic follicles were significantly ($p<0.05$) higher in the EV-treated group compared with the control (Table 1).

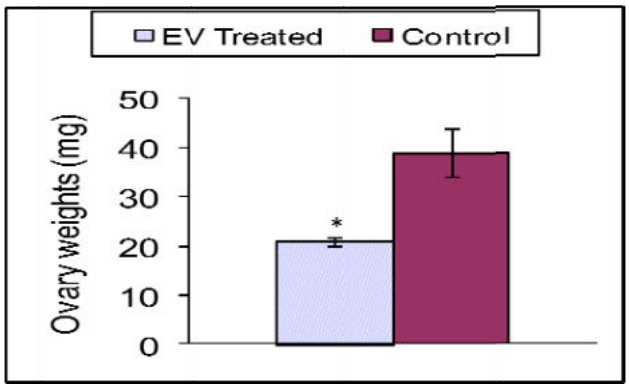

Figure 2 - Ovarian weight measured on Day 30 of EV treatment.

The charts show the ovarian weight of the animals on Day 30 of treatment. EV- Estradiol valerate. Values are expressed as the mean ± SD. n=6. * Value significant at $p<0.01$.

Table 1 - Effect of EV treatment on ovarian morphology at Day 30

Ovarian feature	EV-treated group	Untreated control group
Atretic follicle	3.5±0.37*	0.00±0.00
Cystic follicle	4.87±0.81*	0.0±0.00
Cystic follicle diameter (µm)	49.56±0.88*	0.0±0.00
Cystic follicle thickness (µm)	24.72±2.72*	0.00±0.00

Values are expressed as the mean ± SD. n=6. * Value significantly different from the control at $p<0.05$

The effect of *Ficus platyphylla* on serum hormones, ovarian morphology and weight of ovary of EV-induced PCOS rats

In the 400 mg/kg extract dose, the LH/FSH ratio was the lowest among the tested groups (Table 2). The LH levels in the EV-treated control group were significantly decreased ($p<0.05$) compared with the other extract groups and the CC group. The extract groups showed a dose-dependent increase in the LH levels (Table 2). The 400 mg/kg extract group had significantly higher FSH levels compared with the control and all treated groups. The FSH levels in the CC group were higher than were those of the control group. The estradiol and progesterone levels in the extract groups showed an increase that was consistent with the increase in the extract dose. There was no significant difference in the testosterone level in all the groups ($p<0.05$).

The ovarian weight of the EV control group showed a significant ($p<0.01$) decrease among the groups (Fig 3).

The ovaries of the EV control group exhibited more cystic follicles compared with the other groups ($p<0.05$, Table 3). There were cystic follicles in the 100 mg/kg group, but these were not evident in the 200 and 400 mg/kg groups. Both the 200 and 400 mg/kg groups showed normal follicles at different stages of development. However, there was evidence of atretic follicles present in the 200 mg/kg. The group that received 400 mg/kg showed numerous healthy developing follicles (Figure not shown). The follicular diameter and thickness of the cysts in the PCOS untreated control group were increased compared with those of the 100 mg/kg extract group and the CC-treated group.

Table 2 - Effect of *Ficus platyphylla* on the serum hormones in estradiol valerate-induced PCOS rats

DOSE(mg/kg)	LH	FSH	Estradiol	TSN	PRGSN
100 F.p	5.62 ± 0.81*	2.02 ± 0.01	17.97 ± 11.82	0.02 ± 0.10	57.63 ± 17.18
200 F.p	3.80 ± 0.26*	6.72 ± 1.34*	20.53 ± 9.76*	0.01 ± 0.00*	63.85 ± 3.60
400 F.p	2.47 ± 1.17*	35.92 ± 18.22*	45.96 ± 11.78*	0.01 ± 0.00*	82.89 ± 2.29*
Water	11.97 ± 6.77	2.00 ± 0.76	13.43 ± 9.77	0.02 ± 0.10	63.43 ± 9.65
CC	5.60 ± 1.88*	7.74 ± 2.03*	44.40 ± 12.54*	0.01 ± 0.00*	80.12 ± 2.79*

LH-luteinising hormone (mIU/ml), FSH-follicular stimulating hormone (mIU/ml), Estradiol (pg/ml), PRGSN-Progesterone (ng/ml), TSN-Testosterone (ng/ml). Values are expressed as the mean ± SD. Values significant at $p<0.05$, n=6. * Significantly different from the distilled water control

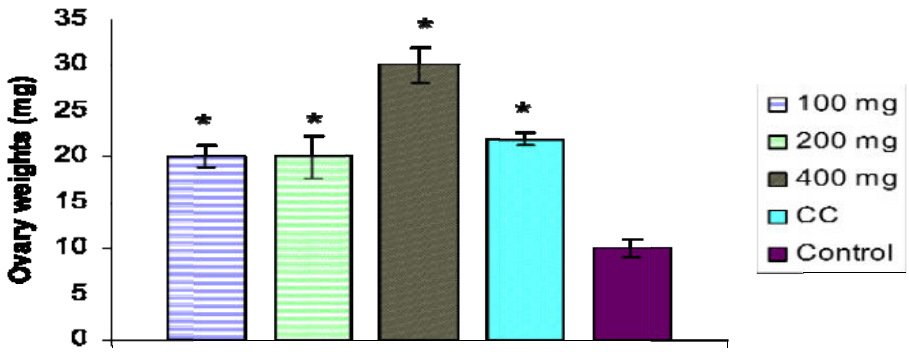

Figure 3 - Effect of *Ficus platyphylla* on Ovarian weight.

Effect of *Ficus platyphylla* on the weight of ovaries of Estradiol valerate-induced PCOS rats. CC=Clomiphene citrate. Values are expressed as the mean ± SD of the rats (n=6). * Value significantly different from other groups at p<0.05.

Table 3 - Effect of *Ficus platyphylla* on the ovarian morphology of the PCO rats

Dose (mg/kg)/ ovarian feature	100	200	400	CC	PCOS control
Atretic follicle	1.5±0.25*	3.13±1.2	0.07±0.06*,a	1.34±0.11*	4.25±0.63
Cystic follicle	4.22±0.21*	0.00±0.00*,a	0.00±0.00*,a	4.30±0.33*	10.1±0.112
CFD (µm)	63.56±8.36*	0.00±0.00*,a	0.00±0.00*,a	77.23±4.95	89.1±9.66
CFT (µm)	36.04±3.05*	0.00±0.00*,a	0.00±0.00*,a	40.33±2.11	45.89±1.04

CC= Clomiphene citrate, CFD= Cystic follicle diameter, CFT= Cystic follicle thickness. Values are expressed as the mean ± SD of the rats (n=6). * Values significantly different from PCOS control group at p<0.05, and [a] significantly different from CC treated group at p<0.05.

Discussion

Many models could be used to study PCOS in humans, but a fully convincing model has not been established [23]. Although the induction of PCOS in rats using EV treatment may not be the best model for studying human PCOS, the anatomy and physiology of the rat ovary resemble those of the human ovary [23]. In the present study, we investigated the effect of the aqueous extract of *Ficus platyphylla* on the serum levels of LH, FSH, estradiol, testosterone and progesterone in EV-induced PCOS in the female *Rattus norvegicus* Wistar strain. We also histologically examined the ovaries of all experimental groups. After 30 days of PCO induction, the animals were analysed both hormonally and histologically. On the 16th day (after 15 days of treatment with the extract) the animals were also analysed irrespective of their oestrus cycle. The untreated PCO group served as the control.

An elevated LH/FSH ratio and anovulation are typical findings in women with PCOS [6, 26]. Because our study showed a dose-dependent reduction in this ratio in the extract groups, it is possible that the extract could reverse PCOS. It is noteworthy that the 400 and 200 mg/kg extract dose groups exhibited a greater reduction in this ratio compared with the CC group. Among other reasons, it may be possible that either the extract is more effective in the treatment of PCOS or that its action does not follow the same mechanism. Ovulatory disorders such as PCOS with high serum LH levels are difficult to treat, and numerous therapeutic regimens have proven unsatisfactory with regard to improving hormone secretion or achieving successful ovulation [27]. These regimens include CC, which has a success rate of approximately 50% [28-30]. In this study, our extract was better than the CC treatment group at reversing this ratio.

Oestrogens, similar to other steroids, become altered in PCO [31]. The alteration of oestrogens leads to the formation of cysts in the ovary [12]. Our study showed that *F. platyphylla* induced an increase in serum estradiol, implying that the plant caused a marked improvement in endocrine function and recovery of ovulatory functions in the rats [27]. Similarly, the histomorphometry of PCO was a suitable measurement for describing the cystic status because differences were observed in the morphological characteristics and in the presence or absence of follicular cysts [25]. The plant demonstrated the ability to reverse PCO in a progressive manner. There were numerous healthy follicles at different stages of development in the extract-treated groups (picture not shown) with a decrease in the number of cystic follicles present. These effects were dose dependent. This result implies that the animals may probably be preparing for ovulation.

Hyperandrogenism (a result of high testosterone levels), which is evident in human PCOS [23, 32], was not present in this animal model of EV-induced PCOS [33]. Therefore, no effect of the extract on androgen levels was observed using this model of PCOS induction. It is possible that other models, such as the use of the non-steroidal aromatase inhibitor letrozole, which shows a

marked elevation of testosterone levels [23], could provide more information on the effect of this plant extract on hyperandrogenism. Decreased progesterone production, which reflects anovulation, is a factor depicted in human PCOS [23]. This effect was also noted in the rats examined in this study. The progesterone levels of the *F. platyphylla*-treated group (400 mg/kg) were elevated, possibly showing that there was repair of the luteal functions [34].

The histology of the ovaries taken from the estradiol valerate-treated rats showed that the resulting PCO resembled the human disease [35]. To determine the effect of *F. platyphylla* on the ovarian cysts, we treated the animals with varying doses of the extract. The ovaries of rats from all treated groups exhibited follicles at all stages of development. In both rats and chronic human PCO, cystic follicles are evident and normal healthy follicles are few [27]. Our study showed the formation of numerous healthy follicles and absence of cystic follicles during treatment with the extract. These structures were not clearly evident in both the negative and positive control groups, suggesting that the PCO could have undergone remission in the rats following treatment with the extract. Furthermore, the dose-dependent decrease in the size of the cystic follicular diameter signifies that this extract may contain some potent compounds that could reverse the development of ovarian cysts. It has been reported that the ovaries of the EV-treated rats were smaller than normal ovaries [12], and our study concurred with these findings.

However, further studies are required to determine the oestrogenic activity of this extract and other possible mechanism through which the extract may act. Additionally, the active component of this extract should be isolated and characterised.

Conclusions

In conclusion, the aqueous extract of *F. platyphylla* effectively reversed the PCO state in EV-induced PCO in rats. Further studies are needed to decipher the mechanism of action of this plant and the component responsible for these actions.

Abbreviations

PCOS: Polycystic ovarian syndrome; EV: Estradiol valerate; PCO: Polycystic ovary; LH: luteinising hormone; FSH: follicular stimulating hormone; CC: Clomiphene citrate

Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

CJU conceived the study, participated in its design, execution and analysis and in the interpretation of the data and drafted the manuscript.

OSB participated in the design, coordination, analysis and interpretation of the data and assisted in proofreading the manuscript for the study.

RUO assisted in the execution of the experiment and revision of the manuscript.

EUE participated in the development of the experiments and critically revised the manuscript.

MOU assisted in the execution of the experiments and manuscript revision.

VUI participated in the development of the experiments and critically revised the
manuscript.

MU participated in the assessment of the microscopy

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