

# The inductive effects of *Centella asiatica* on rat spermatogenic cell apoptosis in vivo

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**Abstract** *Centella asiatica* (L.) Urban has been traditionally used for the treatment of various disease and as a food for thousands of years in various parts of the world including eastern Asia, China and India. The goal of this study was to investigate the effects of *Centella asiatica* aqueous leaf extract on the induction of spermatogenic cell apoptosis in male rats. After lethal dose (LD<sub>50</sub>) assessment of plant extract, rats were divided in five groups. The experimental groups received orally 10, 50, 80 and 100 mg/kg aqueous leaf extract daily for 60 days and the control group received just water. After 60 days, body and testis weight were measured and blood samples were taken from the heart. To evaluate apoptosis and histological changes, tissue samples obtained from rat testes were stained by TUNEL assay and hematoxylin and eosin stain. Results showed that the sperm count, motility, and viability and the number of spermatogenic cells in the seminiferous tubules were significantly decreased compared with the control group. The number of apoptotic germ cells per seminiferous tubule cross-section was significantly increased in the experimental group ( $18.11 \pm 3.5$ ) compared with the control group ( $8.7 \pm 0.81$ ) ( $P < 0.05$ ). Serum testosterone,

follicle-stimulating hormone, and luteinizing hormone levels also showed significant decreases in the experimental groups ( $P < 0.05$ ). There was also a significant decrease in testis weight in experimental groups compared with the control group ( $P < 0.05$ ). It is concluded that *Centella asiatica* has toxicological effects on the reproductive system in male rats and, therefore, it is suggested that leaf extracts of *Centella asiatica* possess antifertility effects in the male rat.

**Keywords** *Centella asiatica* · Apoptosis · Spermatogenic · Rat · Testosterone

## Introduction

In the last few years, a marked decrease in the quality of human semen has been reported [1]. These changes in semen quality are more likely to be due to factors including chemicals, traditional healers and medicinal plants and misuse of drugs [2, 3]. Medicinal plants are commonly prescribed for a multitude of everyday conditions. *Centella asiatica* has been reported to be useful in the treatment of diarrhea, asthma, tuberculosis, inflammation, depression, lupus, and various skin lesions, and also to aid the circulatory system, soothe and minimize varicose veins, stimulate the sex drive, and improve the learning and memory processes. However, *Centella asiatica* has immunomodulatory, sedative, antidepressive, antimicrobial, antiviral, anticonvulsant and analgesic effects [4–7].

*Centella asiatica* (L.) Urban (syn. *Hydrocotyle asiatica* L.) belongs to the Apiaceae family (umbelliferae), and is native in India, northern Australia, Malaysia, Iran, Sri Lanka, Papua New Guinea, and other parts of Asia. *Centella* grows along ditches and in low wet areas; this

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aquatic plant is especially sensitive to pollutants in the water [8].

*Centella asiatica* contains triterpene glycosides such as centellasaponin, alkaloids, volatile and flavonoids compounds, steroids, asiaticoside, scaffeoleoside, phenolic acid, and madecassic acid [9–12]. Asiaticoside is the most major triterpene glycoside in the water extract and it is transformed in vivo by hydrolysis into asiatic acid, which was shown to be effective in induction of apoptosis in different types of cancer [13–15].

Some studies have investigated *Centella asiatica* scientifically and it was found to possess a number of notable pharmacological effects including antipsoriatic [16], wound healing [17], hypoglycaemic [18], hepatoprotective [19], anti-gastric ulcer [20], anti-tumour, antimicrobial [21], antinociceptive and anti-inflammatory [22], antioxidant [23] and anxiolytic properties [24].

Male reproductive function is dependent on the normal status of the male reproductive organ and its accessory organs. The male reproductive organ is the testis, which is primarily responsible for the production of spermatozoa. Sperm production occurs in the seminiferous tubules of the testis, which is controlled by testosterone produced in Leydig cells [25]. Testosterone production is directly dependent on the concentration of luteinizing hormone (LH) secreted by the anterior pituitary gland. Follicle-stimulating hormone (FSH) released by the anterior pituitary stimulates the Sertoli cells which give support and nourishment to spermatozoa through their developmental steps. Therefore the quality and quantity of produced semen depend on normal function of the testicular structures and reproductive hormones [26]. *Centella asiatica* is thought to be medicinally useful; its traditional use in reproductive remedies has been reported: a decoction of plant leaves is widely used in India and eastern Asia as a male contraceptive. The aim of this study was to investigate the effect of a *Centella asiatica* aqueous extract, as an Iranian native plant, on spermatogenic cell apoptosis induction in the male rat.

## Materials and methods

### Plant collection and identification

The fresh leaves of *Centella asiatica* were collected in May 2007 from a local wetland at Bandar-Anzali, Iran. The accuracy of plant selection was proved and authenticated by Dr. Abrahmi, a senior botanist of the Department of Botany, University of Tehran, and a voucher specimen was deposited accordingly at the herbarium of the Department of Plant Sciences at the University of Tehran.

### Extraction

The fresh leaves of the plant were air-dried at 40°C and ground to powder, which was then subjected to exhaustive extraction using ethanol in a Soxhlet apparatus. The dark green liquid extract was concentrated under vacuum and the resulting dried extract was lyophilized and preserved in a refrigerator at 4°C until use in the experiments.

### Experimental animals

The animals used in this study were adult Wistar albino male rats, 8–10 weeks old and weighing  $250 \pm 10$  g, obtained from animal facility of the Pasteur Institute (Tehran, Iran). Male rats were randomly distributed into cages and allowed to acclimatize for 10 days in a well-ventilated room at  $25.0 \pm 2.0^\circ\text{C}$  with constant humidity of 40–70% and a 12-h light/12-h dark cycle prior to experimental protocols. All animals were treated in accordance to the principles of laboratory animal care [27]. Body weight and daily intake of food and water were determined several times per week throughout the study.

### Acute toxicity test

The lethal dosage ( $\text{LD}_{50}$ ) values of *Centella asiatica* extract was evaluated in rats. In this method, rats were randomly divided in four groups (control and experimental groups) that received a single dose of *Centella asiatica* extract (100, 500, 1000, 2000 mg/kg) using intubation needles. All the animals were allowed access to feed and water. The mortality and manifestation rate was recorded during a 24-h period [28].

### Experiment protocol

Forty adult male rats were equally divided in five groups: one control and four experimental groups. During the experiment, the crude extract was dissolved in distilled water and administered orally to rats at a concentration of 10, 50, 80, and 100 mg/kg body weight in 1 ml volume as single daily doses for 8 weeks using animal feeding gavage. Similarly, the controls received gavage of 1 ml of distilled water in the same schedule as the experimental rats. On the sixtieth day of treatment (end of the experiment), the rats were anesthetized using intraperitoneal pentobarbital sodium (50 mg/kg) and were then killed by decapitation according to the recommendation of the Institutional Ethical Committee [29]. Finally, the peritoneal cavity was opened through a lower transverse abdominal incision. The testes were carefully removed, washed in normal saline solution (0.9%), blotted, and weighed. Testes were fixed in Bouin's fixative for assessing morphological

changes and in formaldehyde fixative for TUNEL assays. Tissues were processed using standard procedures.

#### Measurement of semen parameters

The caudal epididymis was immediately dissected and an incision of about 1 mm made. A drop of seminal fluid containing sperm was loaded onto the microscope slide and 2 drops of normal saline were added to mobilize the sperm cells. Epididymal sperm motility was then assessed by calculating motile spermatozoa per microscope field and was expressed as a percentage. In order to count sperm, the epididymis was first homogenized in 5 ml of normal saline. The counting was then done using the counting chamber in the hemocytometer [30]. The sperm viability and morphology were also determined using eosin–nigrosin staining as described previously [31, 32].

#### Measurement of hormones

Blood samples were collected by heart puncture using a syringe into a lithium heparinized bottle, and centrifuged for 15 min at 3000 rpm. The serum obtained was stored at  $-20^{\circ}\text{C}$  for hormone assay. Serum concentrations of FSH and LH were determined in duplicated samples by radioimmunoassay (RIA) with Rat FSH and LH kits (Biocode, Belgium) according to the protocol provided with each kit. The sensitivities of hormone detection per assay tube were 0.2 and 0.14 ng/nl for FSH and LH respectively. Serum concentration of total testosterone was measured by using a double antibody RIA kit (Beckman, USA). The sensitivity of hormone detection per assay tube was 0.025 ng/nl. [33].

#### Histopathological studies

As mentioned earlier, each testis was carefully isolated, weighed, washed in buffered saline and fixed in Bouin's fixative for 1–2 days, and embedded in paraffin and then sectioned at 5  $\mu\text{m}$  thickness. In accordance with standard histological techniques, the sections were stained with hematoxylin and eosin for microscopic evaluation to assess histopathological changes between control and experimental animals [34].

#### Assessment of apoptosis

The in situ detection of cells with DNA strand breaks was carried out by the TUNEL method. The TUNEL assay was performed the same as has been reported previously with some modifications. The choice of fixative was based on the results of previous studies, which showed that glutaraldehyde fixation significantly improved both TUNEL

specificity and sensitivity while maintaining excellent morphological preservation [35–37].

In brief, after deparaffinization and rehydration, tissue sections were incubated with proteinase K for 15 min at room temperature, washed in distilled water, and then treated with 2% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min at room temperature to suppress endogenous peroxidase activity.

The sections were incubated with the TUNEL reaction mixture, fluorescein-dUTP (in situ Cell Death Detection, POD kit, Roche, Germany), for 60 min at  $37^{\circ}\text{C}$ . The slides were then rinsed three times with PBS and incubated with secondary anti-fluorescein-POD-conjugate for 30 min. After three times washing in PBS, diaminobenzidine- $\text{H}_2\text{O}_2$  (DAB, Roche, Germany) was added to sections and counterstained with hematoxylin. As a control for method specificity, the step using the TUNEL reaction mixture was omitted in negative control serial sections, and a nucleotide mixture in reaction buffer was used instead. Apoptotic germ cells were quantified by counting the number of TUNEL-stained nuclei per seminiferous tubule cross-section. Cross-sections of 100 tubules per specimen were assessed and the mean number of TUNEL-positive germ cells per tubule cross-section was calculated.

#### Statistical analysis

The data were statistically analyzed using SPSS software (version 13). The means of the experimental groups were compared with the control group using the ANOVA test and Student's *t* test. Data were expressed as mean  $\pm$  SEM and  $P < 0.05$  was considered as a significant difference.

## Results

#### Acute toxicity test

The oral  $\text{LD}_{50}$  of the extract of *Centella asiatica* was calculated with Pharm/Pcs probit scale versus log dose, value  $r = 0.96$ , standard deviation = 0.5 and regression equation, and found to be 200 mg/kg with a confidence of 1.9–2.2 in male rats. A dose of 100 mg/kg/day showed significant changes in the reproductive system.

#### Body and testes weight

Table 1 shows that administration of extract after 60 days caused a significant increase in the body weight of treated males when compared with the control group. The weights of testes decreased significantly in experimental groups compared to the control group ( $P < 0.05$ ).

**Table 1** The effect of *Centella asiatica* on weight and sperm parameters of control and experimental groups of male rats

	Control	Dose (mg/kg)			
		10	50	80	100
Body weight (g)	284.5 ± 11.2	287.3 ± 9.3	319.2 ± 13.2	327.5 ± 7.9	331.8 ± 12.2
Epididymis weight (g)	0.30 ± 0.025	0.30 ± 0.02	0.26 ± 0.05	0.22 ± 0.08	0.20 ± 0.38
Testis (g)	1.39 ± 0.05	1.30 ± 0.02	1.19 ± 0.04*	0.91 ± 0.03*	0.83 ± 0.09*
Number of sperms from cauda epididymis (×10 <sup>6</sup> )	61.60 ± 2.34	57.7 ± 7.03	44.2 ± 3.8**	39.7 ± 6.2***	36.7 ± 4.8***
Motility (%)	94.3 ± 3.3	72.3 ± 5.4**	54.2 ± 3.9***	47.7 ± 2.4***	35.8 ± 4.3***
Viability (%)	96.2 ± 1.7	87.7 ± 4.03**	84.5 ± 4.4**	51.7 ± 5.09***	47.8 ± 6.01***

Data are presented as mean ± standard error ( $n = 10$ )

Significant differences between control and experimental groups: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$

**Table 2** The effect of *Centella asiatica* on serum levels of LH, FSH and total testosterone of control and experimental groups in male rats

	Control	Dose (mg/kg)			
		10	50	80	100
Serum testosterone level (IU/L)	4.14 ± 0.24	4.3 ± 0.43	3.41 ± 0.29*	2.67 ± 0.49*	2.02 ± 0.29*
FSH level (IU/L)	7.58 ± 0.24	7.3 ± 0.29	7.5 ± 0.29*	7.3 ± 0.32*	6.45 ± 0.05*
LH level (IU/L)	4.01 ± 0.26	3.89 ± 0.2	2.63 ± 0.14**	2.46 ± 0.21*	1.71 ± 0.15**

Data are presented as mean ± standard error ( $n = 10$ )

Significant differences between control and experimental groups: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$

#### Results of sperm count, viability and motility

Table 1 shows that administration of extract after 60 days significantly reduced sperm count and motility in all experimental groups relative to the control group. Moreover, sperm viability was significantly decreased in experimental groups relative to the control group.

#### Results of serum testosterone, LH and FSH hormones

Table 2 illustrates a significant reduction in serum levels of testosterone, FSH and LH relative to the control group.

#### Results of histopathology of testis

Histopathological studies of testis tissue demonstrated a significant decrease in the number of spermatogenic cells (spermatogonia, spermatocyte, spermatid and sperm) in the seminiferous tubules in all experimental groups relative to the control group. Intertubular spaces and venous congestion were increased in the treatment groups compared with those seen in the control group.

The loss in testicular weight likely corresponded to a dose-dependent decrease of mean spermatogenic cells in seminiferous tubules. Sperm density declined significantly in all experimental groups and seminiferous tubules showed degeneration (Fig. 1a–d).

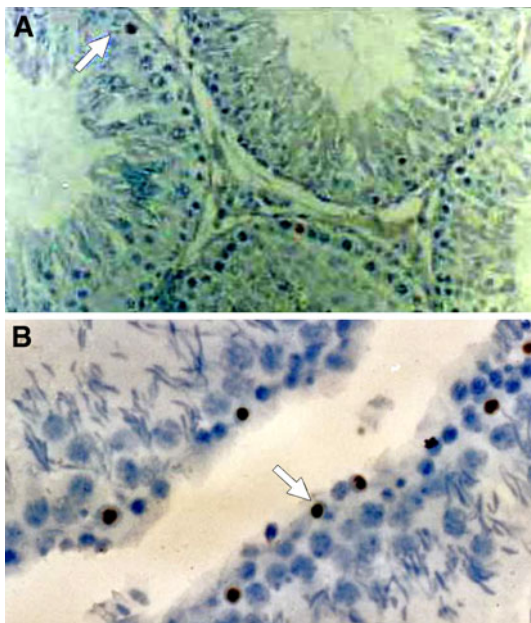
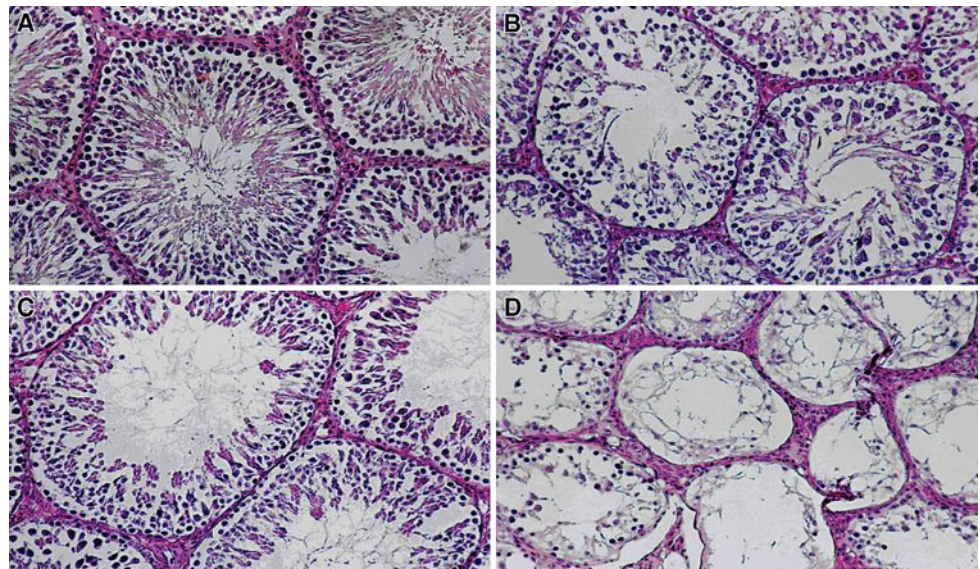
#### Results of apoptotic germ cell assay

In this study, the TUNEL assay showed a number of positively stained spermatogenic cells in experimental groups. The TUNEL-positive spermatogonia and spermatocytes were the main germ cells undergoing apoptosis. The in situ detection method identified apoptotic cells by their darkly stained nuclei. We also observed a low incidence of spontaneous apoptosis in normal rat testis from the control group (Fig. 2a, b). The means of total apoptotic cells per seminiferous tubule cross-section (spermatogonia and spermatocytes) were  $18.11 \pm 3.5$  and  $8.7 \pm 0.81$  in the experimental and the control groups, respectively. Table 3 shows the descending trend of spermatogenic cell survival with increase in extract dose.

#### Discussion

Herbs have been used as important therapeutic agents for a long time. The knowledge of traditional medicine which is inherited from ancestors is very useful for daily life. Some of the herbs are more effective and valuable than those in modern medicine. Many of them have been claimed to be usable as drugs in traditional medicines. Medicinal plants are widely used in therapy because these plants have fewer side effects and lower price than modern drugs. However,

**Fig. 1** **a** Testis tissue in experimental group 10 mg/kg body weight. **b** Testis tissue in experimental group 50 mg/kg body weight. **c** Testis tissue in experimental group 80 mg/kg body weight. **d** Testis tissue in experimental group 100 mg/kg body weight (all H&E staining;  $\times 40$ )



**Fig. 2** **a** Testicular section from a control rat shows apoptosis in primary spermatocyte; dark brown apoptotic body (*arrow*) detected by TUNEL assay ( $\times 40$ ). **b** Testicular section from a rat treated with *Centella* shows apoptosis in primary spermatocyte (*arrow*) detected by TUNEL assay ( $\times 40$ )

there is little scientific data supporting most of their traditional uses, so studies are required to achieve more outcomes in humans and to introduce them into medical use.

The male reproductive system consists of the testis as the main reproductive organ and other accessory structures responsible for sperm production. Agents that alter testicular function will affect the quality and quantity of spermatozoa, which depends on several reproductive factors [38].

The animal model has previously been used to assess the effects of various extracts obtained from medicinal plants on male reproductive function. Since the spermatogenic process in rats lasts 60 days [39, 40], in this study aqueous leaf extracts of *Centella asiatica* were administered for the same period. The present study showed that oral administration of *Centella asiatica* induces rat spermatogenic cell apoptosis and reduces fertility in male rats. As we know, the weight, size and secretory function of testis as reproductive organ is regulated by androgens [41]. Toxins and drugs may affect the pituitary gland and decrease the main process of spermatogenesis. Decreased androgen production is reflected by a decreased number of mature Leydig cells and their functional status. In the present study, the number of degenerating Leydig cells was significantly increased. This may manifest as a decrease in androgen levels. This decrease was further confirmed through a decline in spermatocytes and spermatids, a population which is completely androgen-dependent [42]. The results of this study show that the aqueous extract of *Centella asiatica* impairs reproductive function in the male rat. With regard to the ascending trend of adverse effects on semen parameters and testicular histology in conjunction with dose increase, it clearly appear that the effects of *Centella asiatica* may be mainly due to its direct deleterious impacts on the seminiferous tubules, according to the study by Mahanem and Norazalia [43].

In agreement with previous studies, exposure of the rats to a toxic compound was followed by a weight decrease in androgen-sensitive organs [44, 45]. Weight decrease of reproductive organs in the current study was further confirmed concurrently with the decrease in androgen levels. Researchers have demonstrated that physiological concentrations of testosterone, LH, and FSH play an important

**Table 3** The effect of *Centella asiatica* on testicular cell population in control and experimental groups in male rats

	Control	Dose (mg/kg)			
		10	50	80	100
Spermatogonia	56.9 ± 2.08	55.8 ± 2.1	49.0 ± 2.25*	32.3 ± 2.92**	10.13 ± 1.4**
Spermatocyte	69.0 ± 3.16	61.0 ± 3.0	58.5 ± 2.16*	45.6 ± 3.9**	22.5 ± 2.9**
Spermatid	180.8 ± 9.83	176.5 ± 6.68	159.5 ± 8.9	141.3 ± 9.51*	30.65 ± 1.3**

Data are presented as mean ± standard error. Values are in number/cross-section

Significant differences between control and experimental groups: \* $P < 0.05$ , \*\* $P < 0.01$

role in spermatogenesis [46, 47], so the decrease in the concentration of these hormones in our study could reduce the number and function of somatic and germinal cells in the testis and consequently cause weight reduction of the testes.

The present study showed that administration of *Centella asiatica* for 60 consecutive days results in a marked reduction in sperm count, sperm motility and viability as compared to controls. This is in agreement with previous studies such as Yuniato et al. [48] who reported that *Centella* treatment for 42 days in mice caused in a marked reduction in sperm count and motility. In addition, it has been similarly shown that *Centella asiatica* treatment for four weeks in mice resulted in a significant decline in sperm count and did not affect sperm motility and morphology [49, 50]. The decrease in sperm count in this study is likely due to direct damages to the Leydig and Sertoli cells which are directly involved in the production of spermatozoa.

It has been reported that decrease in sperm count and motility are valid indices of male infertility in laboratory animals [51, 52]. However, sperm motility is often used as a marker of chemical-induced testicular toxicity [53]. It is also stated that the disruption of the seminiferous epithelium is indicative of male reproductive risk. Therefore, our experimental results suggest a gonadotoxic potential of *Centella*. One of the reasons for this effect could be *Centella*'s interaction with optimum energy metabolism for sperm vitality and motility. Apoptosis of germ cells occurs normally in the testis and is essential for the normal maintenance of spermatogenesis, whereas a relatively small increase in the percentage of germ cell apoptosis can result in defective spermatogenesis leading to infertility [45, 54]. Increases in the incidence of germ cell apoptosis are often observed as a result of various forms of physical or chemical injury to the testis [55]. The present study demonstrated that *Centella asiatica* can result in apoptosis in male rat germ cells. These results indicate that *Centella asiatica*, similar to other medicinal plants, may directly interfere in the process of spermatogenesis. The increase in germ cell apoptosis is possibly due to increased peroxide radical generation in the testis following *Centella asiatica*

treatment. Therefore, *Centella asiatica* could also induce apoptotic pathways through activation of caspases. The results indicate that *Centella asiatica* has some effects in the male rat reproductive system. Furthermore, *Centella asiatica* can cause changes in the structure of the testicular histology, characterized by edema and reduced spermatogenesis.

As seen in Table 1, the body weight of the experimental group increased synchronously with decrease in testes weight when the dose changed from 10 to 100 mg/kg. Considering some studies on leptin receptors and leptin expression on germ cells within the testis [56, 57], there is a marked similarity between the outcomes obtained for leptin deficiency and for *Centella asiatica* experimental recommendation in male rat. As Bhat et al. [56] showed, body weights in the leptin-deficient cases are significantly increased while the weights of the testes are reduced as compared to control animals, the same as the present outcome using *Centella asiatica*. Regarding the above likenesses, and also similar inductive changes in endocrinological features such as LH and FSH reduction, and on the other hand the role of leptin changes in spermatogenic cell metabolism or even apoptosis [57], it appears that leptin could be a putative intermediate factor in the body weight increase in the experimental group. In addition, the relationship between sperm hypomotility with leptin deficiency as well as with *Centella asiatica* suggests a parallel regulatory role in reproduction at the level of the male gonad.

The results of the present study suggest that exposure to *Centella asiatica* through oral administration causes male reproductive toxicity in rats. Evidence for this toxicity included degenerative changes in the seminiferous tubules, absence of spermatozoa in the testes, and germ cell apoptosis, likely mediated via a direct deleterious action on the testis without strict disruption of the testicular endocrine function.

In conclusion, this study suggest that *Centella asiatica* has potential testicular toxicity as evidenced by decreased sperm count and motility, increased apoptotic cells, and structural damage to the testis in a rat model. It appears that this herbal remedy could be applicable to male

contraception in humans. Although such herbal contraceptives would be useful if hormonal and surgical methods are contraindicated, many of these plants cannot be recommended for practical use in men due to a lack of comprehensive pharmacological data and knowledge. Therefore, further research is needed to achieve reliable contraceptives derived from these plants that do not induce any side effects.

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