Three Medicinal Plants Affecting Human Ovarian Cell Viability, Hormone Release, and Response to Environmental Contaminant Toluene

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Summary
The present study examined the effect of medicinal plants - ginkgo, tribulus (*puncture vine*), and yucca - on ovarian functions and their response to the toxic influence of toluene. Therefore, we analyzed the effect of toluene with and without these plant extracts on cultured human ovarian granulosa cells. Cell viability and the release of progesterone, insulin-like growth factor I (IGF-I), oxytocin, and prostaglandin F (PGF) were analyzed using the trypan blue test, enzyme immunoassay, and enzyme-linked immunosorbent assay, respectively. The ginkgo, tribulus and yucca were able to suppress ovarian cell viability and alter the release of hormones. Toluene suppressed cell viability and the release of PGF, but not of progesterone, IGF-I, or oxytocin. The negative effect of toluene on cell viability was prevented and even reversed by ginkgo and yucca, whereas its effect on PGF was prevented or inverted by all tested plant extracts. These findings (1) demonstrated the direct toxic effect of toluene on ovarian cells, (2) showed the direct effect of some medicinal plants on ovarian cell functions, and (3) demonstrated the ability of these plants to inhibit the effects of toluene and to act as natural protectors against the suppressive effect of toluene on female reproduction.

Key words
Toluene • Plants • Ovary • Cell viability • Hormones

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Introduction

Medicinal plants can be utilized as cheap and safe drugs to prevent or treat reproductive disorders and as those that affect reproductive processes. For instance, ginkgo (*Ginkgo biloba* L.) can increase rat ovarian weight, suppress proliferation and progesterone release, and promote apoptosis in cultured porcine ovarian cells. However, its extract can also be used to prevent and treat ovarian cancer, ischemia, and menopausal syndrome [1,2].
Puncture vine (*Tribulus terrestris* L.) can reportedly promote both proliferation and apoptosis of porcine ovarian cells [3]. It can also be used to treat symptoms of polycystic ovarian syndrome and infertility in women [4].
Yucca (*Yucca schidigera* Roezl ex Ortgies) has been shown to promote rabbit ovarian folliculogenesis and fecundity [5,6]. Consumption of yucca increased the plasma levels of progesterone (P), oxytocin (OT), and prostaglandin F (PGF) in rabbits. Furthermore, the addition of yucca extract to cultured rabbit ovarian fragments promoted the release of P and PGF and suppressed the release of IGF-I, but it did not suppress the release of OT [5,6]. Similarly, the addition of yucca extract to cultured murine ovarian fragments promoted the release of P but did not promote the release of IGF-I [7]. In contrast to findings obtained in rabbits and mice, in ewe, dietary intake of yucca suppressed ovarian folliculogenesis via the promotion of ovarian cell apoptosis. Moreover, the addition of yucca to cultured bovine ovarian fragments suppressed the release of P and IGF-I [8].
Oil-related environmental contaminants, including toluene, which is an aromatic hydrocarbon, can cause many female reproductive and nonreproductive disorders [9-11]. *In vivo,* toluene has been shown to impair gonadotropin-releasing hormone (GnRH) and gonadotropin production, ovarian folliculogenesis, and embryogenesis in animals [11-13]. Recent experiments conducted *in vitro* have demonstrated the direct effect of toluene on ovarian cells. Toluene was found to decrease proliferation in cultured bovine ovarian granulosa cells [14]. Toluene was also shown to reduce ovarian cell viability, proliferation, release of OT and estradiol, and it promoted the release of P and PGF; however, it did not affect apoptosis of cultured porcine granulosa cells [2,3,15]. It reduced proliferation and apoptosis as well as autophagy in rat ovarian granulosa cells [13]. In cultured mare granulosa cells, toluene was shown to inhibit cell viability, proliferation, apoptosis, and the release of P, PGF, and IGF-I; however, it did not inhibit the release of OT [16]. Compounds that can efficiently protect cells against the adverse effects of toluene on reproduction remain unknown at present. Fennel (*Foeniculum vulgare*) is a medicinal plant that has been shown to mitigate some (but not all) effects of toluene on cultured mare granulosa cells [16]. The protective effects of other medicinal plants against toluene ave not yet been studied.
Altogether, the available literature demonstrates the ability of ginkgo, tribulus, and yucca to affect animal ovarian functions *in vivo* and *in vitro* and to treat some reproductive disorders. Furthermore, it shows that toluene has adverse effects on female reproductive processes in animals *in vivo* and *in vitro* and that these effects could be principally

mitigated by the use of plant extracts. Alternatively, the effect of ginkgo, tribulus, and yucca, as well as of toluene, on healthy human ovarian cells has not been determined yet. Furthermore, whether ginkgo, tribulus, or yucca can mitigate the effects of toluene on ovarian cells is unknown; in other words, whether these medicinal plants can be useful as natural protectors against the toxicity of toluene is not known.
This study aimed to (a) determine the effect of toluene on human ovarian cell functions, (b) understand whether and how ginkgo, tribulus, and yucca can directly affect these functions, and (c) examine whether these medicinal plants can mitigate or prevent the effect of toluene. Therefore, we examined the effect of toluene, extracts of ginkgo, tribulus, and yucca, in addition to the effect of the combination of toluene with these plant extracts on cultured human ovarian granulosa cells. Following these treatments, we analyzed cell viability and the release of key ovarian hormones, P, IGF-I, OT, and PGF, which are considered both markers and regulators of ovarian cell functions [17].

Materials and Methods

Isolation and culture of granulosa cells

Ovarian granulosa cells were harvested 1–5 days after spontaneous ovulation from women at the age range of 36–42 with normal ovarian cycles and morphology who were undergoing ovariectomies due to non-metastatic cancer of the cervix uteri. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all patients in accordance with the EU and Slovakian ethical and medical regulations under the supervision of the local ethics committee governing the University Hospital of Nitra, Nitra, Slovakia, where the patients were undergoing treatment. We isolated and processed the granulosa cells (30–50 × 10^6 per ovary) from ovarian follicles 3-5 mm in diameter without visible signs of atresia as described previously [18]. Immediately after isolation, the granulosa cells collected at a final concentration of 10^6 cells/ml were precultured in sterile Dulbecco's modified Eagle's medium /F12 1:1 medium supplemented with 10 % fetal calf serum (both from BioWhittakerTM, Verviers, Belgium) and 1 % antibiotic–antimycotic solution (Sigma, St. Louis, MO, USA) in 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 µl/well) for 3 days. Thereafter, the medium was replaced with a medium of the same composition with and without toluene (AppliChem GmbH, Darmstadt, Germany; 0, 0,1 or 1,0 µg/ml), ginkgo (Xi´an Xuhuang Bio-Tech Chemical Industry Testing Co., Ltd, Shangai, China), puncture vine/tribulus (Changsha Sunfull Bio-Tech., Co., Hunan, China), and yucca (KONFIRM spol. s.r.o., Brno, Czech Republic) each at 10 µg/ml or combinations of toluene (0, 0,1 or 1,0 µg/ml) + one of the plant additives listed above (10 µg/ml). These doses correspond to the doses of toluene that could be available in environmental waste water [19] and the effective doses of toluene [14-16] and extracts of ginkgo [1], tribulus [3], and yucca [5-8] used in previous *in vitro* experiments. Plant extracts were first dissolved in 50 µl dimethyl sulfoxide (DMSO) to obtain a stock solution of 1 mg/ml plant extract. After these stock solutions were dissolved in a culture medium immediately before their addition to the cells, the final concentration of DMSO did not exceed 0.001 %. Studies have demonstrated that DMSO at this dose does not affect cell viability (not shown). Controls included ovarian cells cultured in the incubation medium (with 0.001 % DMSO) without undergoing any treatment and medium incubated without cells (blank control). After two days of culture, the cells and incubation medium were analyzed as described below.

Cell viability test

Cell viability was evaluated using the trypan blue exclusion test as described by Strober [20]. Briefly, the medium from the culture plates was removed after incubating the granulosa cells. Subsequently, the cell monolayer was subjected to trypan blue staining (Sigma Aldrich, Hamburg, Germany) for 15 min. Following removal of this dye, the plates were washed twice with a physiological solution (0.9 % NaCl) and subjected to microscopic inspection (magnification: 400×). The ratio of dead (stained) cells to the total cell count by using of hematocytometer was calculated.

Immunoassay of hormones

The levels of P, OT, and PGF were measured using enzyme immunoassay (EIA) according to Prakash *et al.* [21] as previously described [3,14,16,22] using a plate reader (Multiscan EX, Labsystem, Finland) to measure absorbance at a wavelength of 450 nm.
The levels of IGF-I were determined by enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (LDN Immunoassays and Services, Nodhorn, Germany) according to the manufacturer's instructions. The characteristics of the assays have been described in detail in our previous studies [3,14-16].
All assays were validated on the culture medium samples by dilution tests.

Statistical analysis

Each experiment was repeated three times using different patients (2-3 per experiment). In some experiments, all treatments were tested at once, whereas in others, the effects of only some additives were investigated. The presented data summarize the results obtained from at least three independent experiments. Each experimental group was represented by four culture wells. After EIA and ELISA, blank control values were subtracted from the value determined in cell-conditioned medium to exclude any nonspecific background (<10 % of the total values). Secretion rates were calculated per 10^6 cells/day. Differences between groups were evaluated using Shapiro–Wilk normality and

Student's *t*-tests. SigmaPlot v.11.0 (Systat Software, GmbH, Erkhart, Germany) was used for conducting statistical analysis. Values are presented as mean ± SEM. Differences were considered statistically significant at $p \leq 0.05$.

Results

Toluene affected some but not all functions of cultured human ovarian granulosa cells. When added alone, toluene decreased the viability of human ovarian granulosa cells at all doses added (Fig. 1A,B,C). There was no significant effect of toluene on the release of P (Fig. 2A,B,C), IGF-I (Fig. 3A,B,C), or OT (Fig. 4A.B.C). In contrast, it suppressed the release of PGF at all doses added (Fig. 5A,B,C).
Plant extracts, when added alone, also affected some ovarian cell functions. When added alone, the ginkgo extract suppressed ovarian cell viability (Fig. 1A), release of P (Fig. 2A), IGF-I (Fig. 3A), but it did not suppress the release of OT (Fig. 4A) or PGF (Fig. 5A) (refer to toluene at dose 0 µg/ml).
Similarly, the tribulus extract impaired cell viability (Fig. 1B), the release of P (Fig. 2B) and PGF (Fig. 5B); however, it promoted the release of IGF-I (Fig. 3B) but did not affect the release of OT (Fig. 4B).
The extract of yucca reduced granulosa cell viability (Fig. 1C) and the release of P (Fig. 2C) or PGF (Fig. 5C). It promoted the release of IGF-I (Fig. 3C), but it did not affect OT output (Fig. 4C).
Moreover, plant extracts were found to prevent and in fact reversed the adverse effects of toluene on ovarian cell functions. Ginkgo (Fig. 1A), tribulus (Fig. 1B), and yucca (Fig. 1C) prevented the inhibitory effect of toluene on cell viability. In the presence of yucca, toluene was not inhibited; however, it increased cell viability (Fig. 1C). Toluene did not affect the release of P, IGF-I, or OT. The presence of ginkgo (Fig. 2A) and tribulus (Fig. 2B) induced the suppressive action of toluene on the release of P, whereas in the presence of yucca (Fig. 2C), toluene promoted the release of P. The lack of toluene effect on the release of IGF-I was noted both in the absence and the presence of plant extracts (Fig. 3A,B,C). The presence of ginkgo induced toluene to suppress the release of OT (Fig. 4A), whereas in the presence of yucca, toluene promoted the release of OT (Fig. 4C). In the presence of tribulus, no effect was observed on the release of OT (Fig. 4B). The addition of ginkgo (Fig. 5A), tribulus (Fig. 5B), or yucca (Fig. 5C) completely prevented the suppressive action of toluene on the release of PGF. Moreover, in the presence of tribulus (Fig. 5B) or yucca (Fig. 5C), the effect of toluene was not suppressed; it in fact considerably promoted the release of PGF.

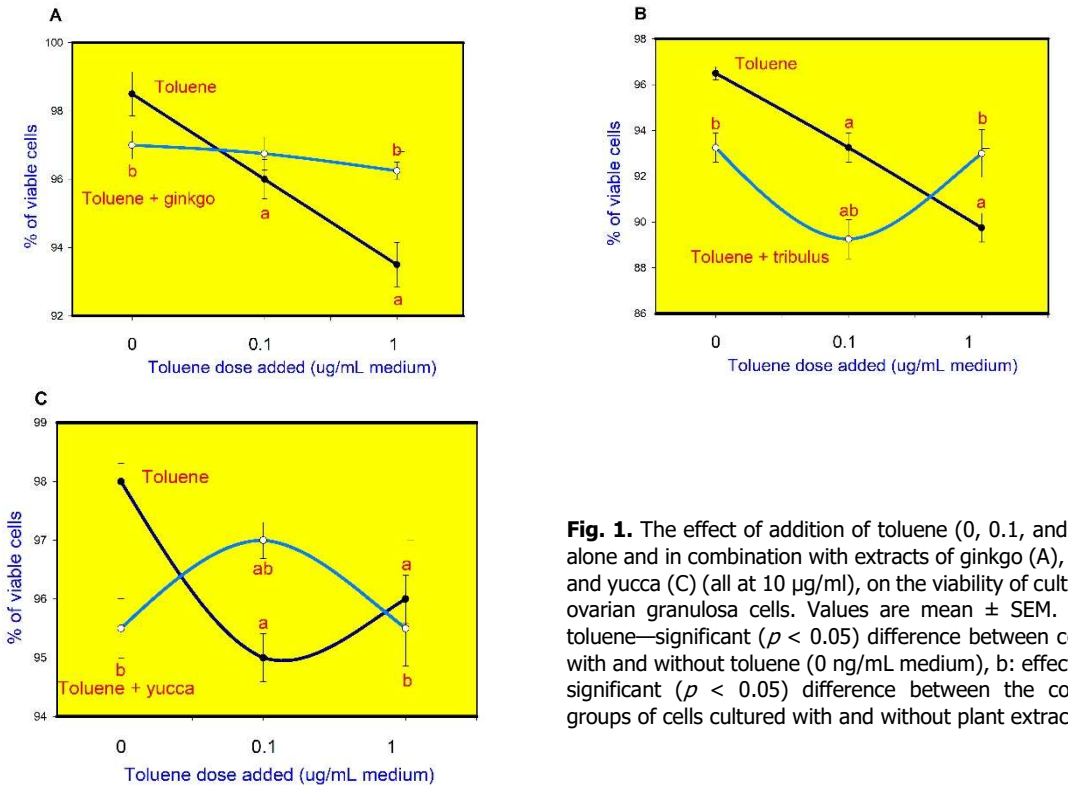


Fig. 1. The effect of addition of toluene (0, 0.1, and 1.0 µg/ml), alone and in combination with extracts of ginkgo (A), tribulus (B), and yucca (C) (all at 10 µg/ml), on the viability of cultured human ovarian granulosa cells. Values are mean ± SEM. a: Effect of toluene—significant ($p < 0.05$) difference between cells cultured with and without toluene (0 ng/mL medium), b: effect of plants—significant ($p < 0.05$) difference between the corresponding groups of cells cultured with and without plant extracts.

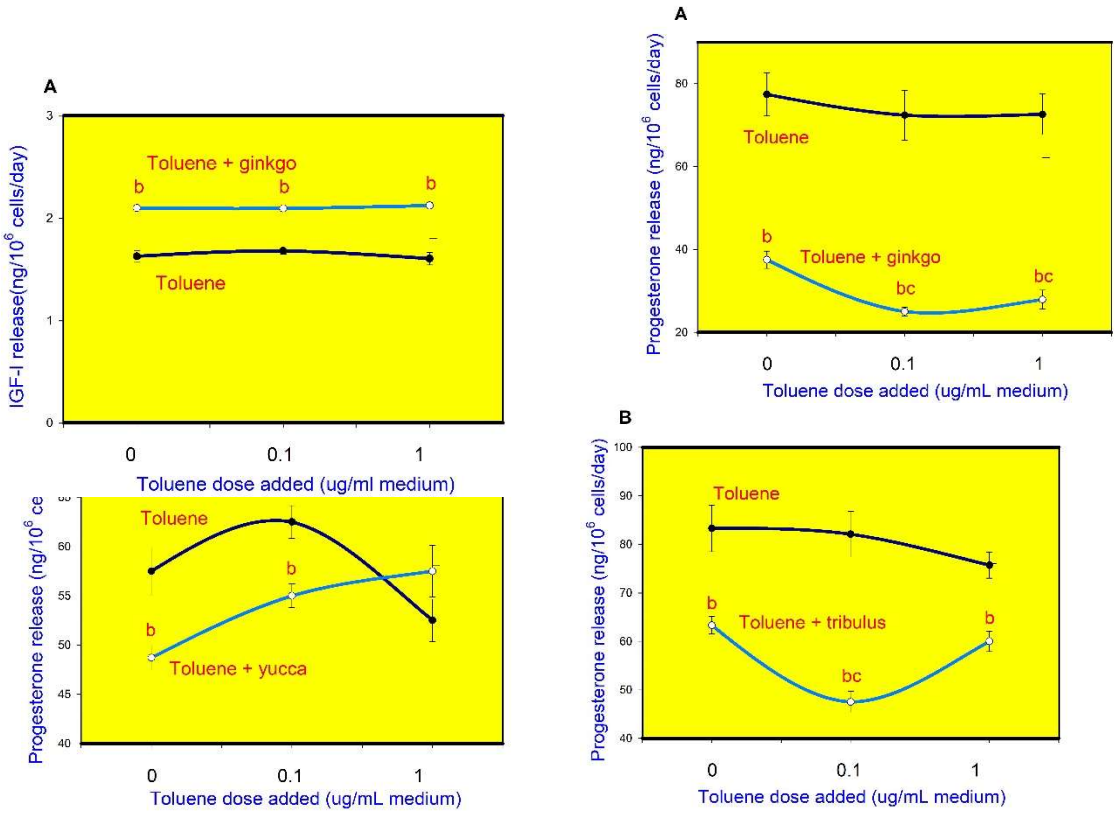
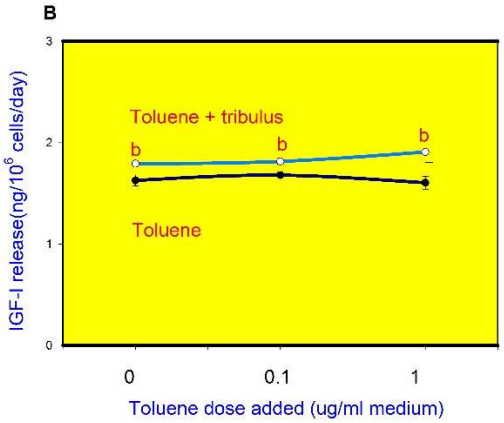


Fig. 2. The effect of addition of toluene (0, 0.1, and 1.0 µg/ml), alone and in combination with extracts of ginkgo (A), tribulus (B), and yucca (C) (all at 10 µg/ml), on the release of progesterone in cultured human ovarian granulosa cells. Values are mean ± SEM. a: Effect of toluene—significant ($p < 0.05$) difference between cells cultured with and without toluene (0 ng/mL medium), b: effect of plants—significant ($p < 0.05$) difference between the corresponding groups of cells cultured with and without plant extracts.



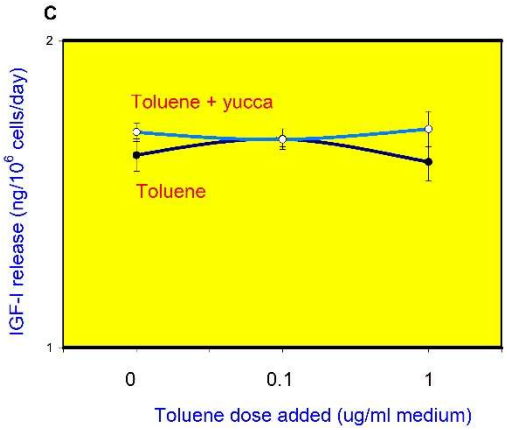
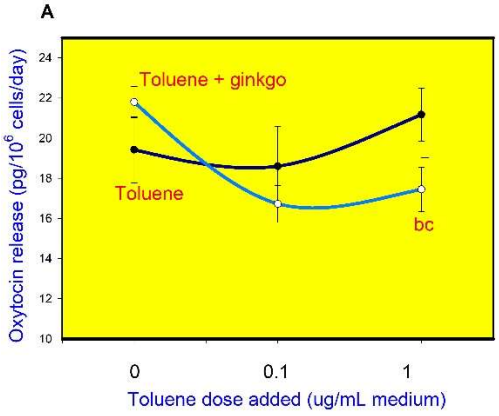
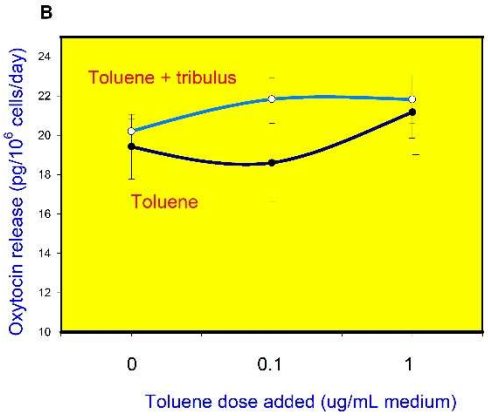


Fig. 3. The effect of addition of toluene (0, 0.1, and 1.0 µg/ml), alone and in combination with extracts of ginkgo (A), tribulus (B), and yucca (C) (all at 10 µg/ml), on the release of insulin-like growth factor I (IGF-I) in cultured human ovarian granulosa cells. Values are mean ± SEM. a: Effect of toluene—significant ($p < 0.05$) difference between cells cultured with and without toluene (0 ng/mL medium), b: effect of plants—significant ($p < 0.05$) difference between the corresponding groups of cells cultured with and without plant extracts.





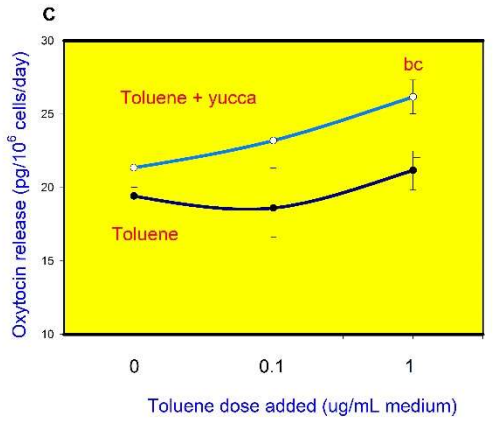
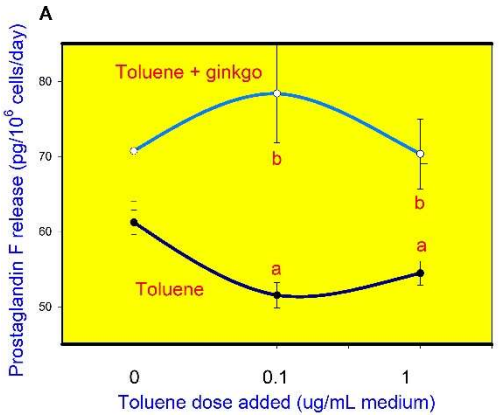
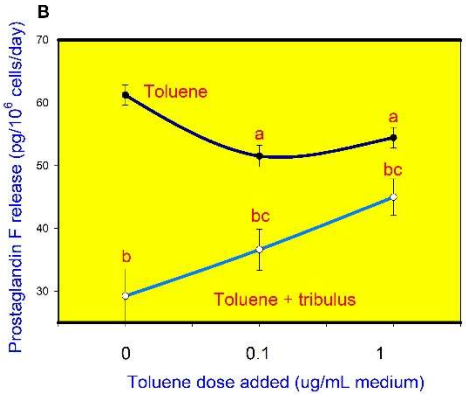


Fig. 4. The effect of addition of toluene (0, 0.1, and 1.0 µg/m), alone and in combination with extracts of ginkgo (A), tribulus (B), and yucca (C) (all at 10 µg/ml), on the release of oxytocin in cultured human ovarian granulosa cells. Values are mean ± SEM. a: Effect of toluene—significant ($p < 0.05$) difference between cells cultured with and without toluene (0 ng/mL medium), b: effect of plants—significant ($p < 0.05$) difference between the corresponding groups of cells cultured with and without plant extracts.





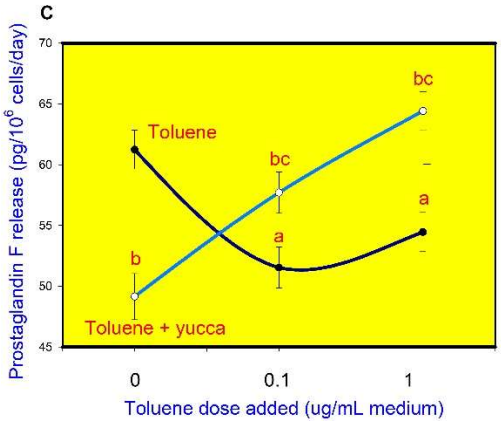


Fig. 5. The effect of addition of toluene addition (0, 0.1, and 1.0 μg/ml), alone and in combination with extracts of ginkgo (A), tribulus (B), and yucca (C) (all at 10 μg/ml), on the release of prostaglandin F in cultured human ovarian granulosa cells. Values are mean ± SEM. a: Effect of toluene—significant ($p < 0.05$) difference between cells cultured with and without toluene (0 ng/mL medium), b: effect of plants—significant ($p < 0.05$) difference between the corresponding groups of cells cultured with and without plant extracts.

The character of influence of toluene, medicinal plant extracts, as well as the ability of plant extracts to modify toluene effect have been summarized in Table 1.

Table 1. Character of effect of toluene, ginkgo, Tribulus terrestris and yucca given alone on cultured human ovarian granulosa cell functions, as well as the ability of ginkgo, Tribulus terrestris and yucca to modify toluene effects

Treatment	Parameter				
	Viability	Progesterone release	IGF-I release	Oxytocin release	Prostaglandin F release
Action of toluene alone	-	0	0	0	-
Action of ginkgo alone	-	-	-	0	0
Action of ginkgo on toluene effect	-	+	0	+	-
Action of Tribulus terrestris alone	-	-	+	0	
Action of Tribulus terrestris on toluene effect	-	+	0	0	-
Action of yucca alone	-	-	0	0	-
Action of yucca on toluene effect	-	+	0	+	-

+ - stimulation or induction, 0 – no effect, - - inhibition.

Discussion

The results obtained in this study demonstrate the effect of plant extracts, toluene, and their combinations on basic cell functions.

Can the tested plants directly affect ovarian functions?

The present study is the first to demonstrate the direct effect of the extracts of ginkgo, tribulus, and yucca on human ovarian cells. In our experiments, the tested plant extracts were responsible for decreasing ovarian cell viability and the release of P. In addition, ginkgo and tribulus promoted the release of IGF-I, and tribulus and yucca suppressed the release of PGF.

The findings of this study in terms of the effect of plant extracts on human ovarian cell functions are consistent with those of previous studies on the action of these plants on cultured animal ovarian cells. Similar to the observation made in case of human ovarian cells in our experiments, yucca [1] and tribulus [3] extracts could suppress the release of

P, cell proliferation, and promote apoptosis in porcine granulosa cells, which could thus suppress porcine granulosa cell viability [1]. The effect of yucca extract on the release of P, IGF-I, OT, and PGF in rabbit [5,6], murine [7], and ovine [8] cells has been studied. The same has also been studied in ovarian cells *in vivo* and *in vitro*. However, the characteristics of yucca action on human and animal cells were different. In contrast with human granulosa cells, yucca promoted the release of P and PGF and suppressed the release of IGF-I; however, it did not promote the release of OT in cultured rabbit ovarian fragments [5,6], promoted the release of P (but not the release of IGF-I) in murine ovarian fragments [7], and inhibited the release of both P and IGF-I in ovine ovarian fragments [8]. These findings demonstrate the substantial differences in the characteristics of plant extract activity on ovarian cells in different species. These variations may be explained on the basis of differences in the dominant mechanisms of action of plants on ovaries in different species. For instance, all tested plants affected hormones (P, IGF-I, OT, PGF), which are considered key regulators of reproductive functions [17]. The species-specific and individual differences in response to plants could be due to differences in the response of the endocrine system to these plants. Furthermore, all tested plants contain several phytoestrogens and antioxidants [2,4,23], which can either upregulate and downregulate steroid hormone receptors and reactive oxygen species, whose number and activity can be species-dependent [12]. Finally, the response of ovarian cells to plant extracts could be affected by their metabolic and hormonal status. For instance, obesity can affect the response of murine ovarian cells to yucca [7]. The addition of ghrelin, a metabolic hormone, can define the response of porcine ovarian cells to tribulus [3].

If the suppressive effect of all tested plants on ovarian cells would be confirmed by future *in vivo* and/or clinical studies, the consumption of these medicinal and functional food plants as well as such plant-containing products and drugs could be considered as harmful to reproductive processes. Such possible side effects should be considered during the application of these plants in human and animal nutrition and phytomedicine. In contrast, the cytotoxic effects of these plants could be useful for their application in preventing and treating ovarian and non-ovarian cancer [2,4,23]. Finally, these medicinal plants may be applicable for the prevention and treatment of reproductive dysfunction induced by environmental contaminants (see below).

Can toluene directly affect ovarian functions?

This study demonstrated the ability of toluene to reduce ovarian cell viability and the release of PGF. This is the first direct demonstration of the effect of toluene on human ovarian cells. It suggests that toluene can suppress female reproductive functions not only via the GnRH/gonadotropin axis [11,12] but also through a direct effect on ovarian cells. These observations are consistent with the results of previous *in vitro* studies, which demonstrated the ability of toluene to reduce the viability of cultured mare [16] and porcine [2] granulosa cells as well as rat granulosa cells *in vivo* [13]. Moreover, they comply with the finding that toluene can downregulate the release of PGF in cultured equine granulosa cells [16]. The reduction in ovarian cell viability could be because of the ability of toluene to downregulate ovarian cell proliferation and/or upregulate their apoptosis. Toluene inhibited the proliferation of cultured bovine [14], mare [16] and porcine [2] granulosa cells. It also inhibited cell proliferation as well as induced apoptosis and autophagy in rat ovarian cells *in vivo* [13].

Alternatively, these studies demonstrated the species-specific effect of toluene on ovarian hormones: in these experiments on human ovarian cells, toluene suppressed only the release of PGF, but it did not suppress the release of P, IGF-I, or OT. In cultured equine granulosa cells, toluene inhibited the release of P, PGF, and IGF-I, but it did not inhibit the release of OT [16], and in porcine ovarian cells, it even promoted the release of P and PGF and suppressed the release of OT [2,15]. The differences in the endocrine effects of toluene could be caused by species-specific differences in the basic production of hormones, e.g., gonadotropins. At least in the presence of follicle-stimulating hormone, the stimulatory effect of toluene on the release of P and PGF by porcine granulosa cells changed to inhibitory effect [15]. Metabolic/body condition state could define the response to toluene too. Toluene showed different effects on markers of proliferation, apoptosis, and the release of P in granulosa cells isolated from the ovaries of cows with different body conditions [14].

These observations are the first direct demonstration of the adverse effect of toluene on female reproductive processes in humans, which was previously hypothesized only on the basis of epidemiological studies, animal, and *in vitro* experiments (see Introduction).

Can some plants modify the effects of toluene on ovarian functions?

To understand the mechanisms of the effects of toluene on ovarian cell proliferation, apoptosis, and secretory activity, further studies are warranted. However, it is hypothesized that toluene can induce apoptosis through its effect on hormones, steroid hormone receptors, and DNA methylation [11], as well as by destroying cell membranes and promoting auto oxidative processes [24]. The presence of high amount of phytoestrogens and antioxidants in ginkgo [2], tribulus [4], and yucca [23] could explain their ability to prevent toluene-induced oxidative stress and changes in steroid hormone receptors by plant extracts.

Previously, such protective effect against some adverse effects of toluene on equine ovarian cells has been described for fennel [16]. The present study demonstrated the ability of ginkgo, tribulus, and yucca to protect the human female reproductive system against adverse effects of toluene for the first time. Such protective effects of these plants suggest their principal applicability for mitigation, prevention, and treatment of reproductive and non-reproductive disorders induced by environmental contaminant(s). Nevertheless, the protective effects of these plants and their safety should be validated by further animal and clinical studies. Furthermore, which compounds could be responsible for imparting protective effects on plants remains unknown. The possible plant constituents which could be responsible for these effects could be apigenin, quercetin, isoquercitrin and resveratrol. At least, these molecules are present in the studied plants, and the protective effects of these compounds against adverse effects of oil-related and other environmental contaminants have been documented [25]. Selection of the most promising plant and its constituents requires further comparative studies. Finally, the mechanisms of plant effects require further elucidation. The similar effect of all three tested plant extracts on ovarian cell viability, the release of P, and effects of toluene indicate similar mechanisms/mediators of their action on ovarian cells. Alternatively, differences in the effect of different plant extracts on release of IGF-I and PGF suggest differences in their endocrine and receptory/postreceptory signalling mechanisms of action.

Such studies could enable better understanding and elicit the application of these medicinal plants for controlling the female reproductive processes, preventing, and treating reproductive disorders, including those induced by toluene.

Authors contribution statement

AS – conceptialization, funding, supervision, writing the manuscript draft; AT, JK, MM – investigation, resources, sample analysis, data curation; SA, SN, AHH - funding, data curation, editing the manuscript.

Data availability statement

The datasets generated during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

There is no conflict of interest.

Informed consent

Informed consent was obtained from all patients in accordance with the EU and Slovakian ethical and medical regulations under the supervision of the local ethics committee governing the University Hospital of Nitra, Nitra, Slovakia.

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