

# *Teucrium polium* plant extract provokes substantial cytotoxicity at the early stage of embryonic development

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## ABSTRACT

The aim of this study is to explore the outcome of *Teucrium polium* (TP) medicinal plant consumption on the early stage of fetal development. We used the chicken embryo at 3 days of incubation as a model to evaluate the effect of TP plant extract during embryogenesis. In addition, quantitative polymerase chain reaction (qPCR) was applied to explore the expression of six genes related to cell proliferation, apoptosis, survival, angiogenesis, and migration. Our data revealed that TP exposure inhibits angiogenesis of the chicken embryo and its chorioallantoic membrane. In addition, we found that TP extract significantly harms the normal development of the embryos since around 95% of TP-exposed embryos died after 1–3 days of treatment. Macroscopic examination did not show any anomalies in these embryos. However, qPCR analysis of activation transcription factor-3, B-cell lymphoma-2, caspase-8, inhibin subunit beta A, vascular endothelial growth factor-C, and Cadherin-6 type-2 genes revealed that these genes are considerably deregulated in heart and brain tissues from TP-exposed embryos in comparison with their matched tissues from unexposed ones. Our study implies that TP plant can have very toxic effects on the early stage of the embryo. Therefore, it is important to alert expectant women to avoid the use of this medicinal plant during pregnancy.

KEY WORDS: *Teucrium polium*; medicinal plant; embryo; angiogenesis; toxicity; gene deregulations

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## INTRODUCTION

The medicinal use of plants dates to ancient times. *Teucrium polium* (TP) is a widespread flowering plant that has been used for over 2000 years in traditional medicine in several regions worldwide, especially in the Middle East area as well as Mediterranean countries [1].

Earlier phytochemical reports have pointed out that TP plant extract contains numerous types of flavonoids such as salvigenin, cirsiolol, luteolin, diosmetin, apigenin, rutin, cirsiarinin, and eupatorin [2,3]. In addition, steroidal composites such as cholesterol,  $\beta$ -sitosterol, stigmasterol, brassicasterol, and campesterol were found in different parts of TP [3,4]. More recently, it has been shown that the oil of TP can contain around 106 different compounds, and the main composites are  $\alpha$ -pinene, cis-verbenol, and myrtenal [5].

Conventionally, TP plant extract was used to treat different types of human health disorders; mostly, it was known as hypoglycemic, hypolipidemic, anticonvulsant, and insulinotropic [1]; it was also used to treat ulcer and inflammation [6,7]. Recently, it has been shown that TP exerts anticancer activities [8]. In this regard, our group has demonstrated that TP extract can inhibit cell invasion and metastasis abilities of human prostate cancer cells through the restoration of E-cadherin/catenin complex; in addition, we reported that it could provoke a massive apoptosis in two human lung cancer cell lines [9,10]. On the other hand, it has been pointed out that TP extract can induce toxicity in human liver tissue and probably other tissues and organs [11,12]. Accordingly, we hypothesized that pregnant women could be very sensitive to this kind of medicinal plants, especially at the early stages of pregnancy. Meanwhile, it is important to highlight that the outcome of TP extract on the normal development of the embryo has not been explored yet. Thus, we herein investigated the effect of TP extract on the early stage of embryogenesis using the avian embryo as a model. Our study revealed that TP extract can have dramatic effects on the embryo at this stage of its development.

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## MATERIALS AND METHODS

### Plant materials

The aerial parts of TP were collected from Al-Raqqa, Syria. The herbs were dried in shade and stored in a dark container. The herbs were identified by Dr. Amal Alachkar from the Faculty of Pharmacy of Aleppo University and authenticated by Dr. Ala-Eddin Al Moustafa who is a coauthor of this paper.

### Extract preparation

Preparation of the extract was performed as follows: Briefly, 3 g of plant material were boiled in 100 ml of autoclaved water for 20° min in a covered beaker. The solution was then filtered using 0.45-µm filter as described previously by our group [9,10]. 50 ml of the extracts were deposited on a round coverslip of 0.5 cm<sup>2</sup> and turned directly onto the chorioallantoic membranes (CAM) of the embryo. Fresh extracts were prepared for each experiment.

### Embryo and in ovo TP plant extract exposure

White Leghorn chicken embryos at 3 days of incubation were used in this assay; this analysis was executed as previously illustrated by our group [13,14]. As mentioned above, 50 µl of TP plant extract or water (as control) was deposited onto CAM of the embryo. On the other hand, *Elaeagnus angustifolia* (EA) medicinal plant extract was also used as control for embryotoxicity [14]. Afterward, TP-exposed embryos and their CAMs as well as their matched controls were examined using stereomicroscope every day for 4 days. Several embryos were sacrificed at 4–7 days of incubation and autopsied. Small pieces of brain and heart tissues were removed for RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

### RNA isolation and quantitative PCR analysis

Total RNAs from heart and brain tissues were isolated using RNA extraction kit (QIAGEN Canada Inc., ON, Canada) according to the manufacturer's protocol. Based on our previous study regarding embryotoxicity, the B-cell lymphoma-2 (BCL-2), caspase-8 (CASPAS-8), activation transcription factor-3 (ATF-3), inhibin beta-A, Cadherin-6 type-2, and vascular endothelial growth factor (VEGF) genes were selected for our investigation [15,16]. Thus, PCR amplification was performed using primer sets for these genes and glyceraldehyde 3-phosphate dehydrogenase gene was used as control. The amplification reactions were carried out with the Applied Biosystems 7500 Fast RT-PCR tool (Applied Biosystems, Waltham, MA, USA). The comparative  $\Delta\Delta C_t$  method was used for relative quantification of the amount of messenger RNA (mRNA) in

each sample once they were normalized to the control. Genes' expressions were calculated relative to control embryo tissues mRNA levels. Standard deviation was expressed as percentage of Ct values of three independent experiments.

### Statistical analysis

The number of deaths of TP-exposed embryos was compared with their matched controls using Chi-square test. In parallel, Kaplan–Meier survival curves of the two groups were generated and compared using log-rank test. SPSS 64-bit version 23 was applied to carry out the previous tests where probabilities <0.05 were considered statistically significant.

## RESULTS

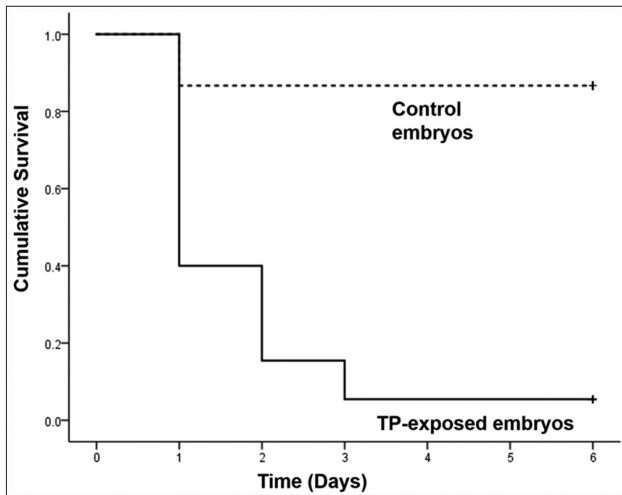
To explore the outcome of TP extract on the embryo at the early stage of development, we evaluated its effect on the chicken embryo at the 3<sup>rd</sup> day of incubation. 110 embryos were exposed to TP extract, as described in the method section; in parallel, 30 embryos were used as controls and exposed to water and/or medicinal plant, EA [14], which was shown to have no harmful effects on the embryo. While examining the embryos daily, we found that approximately 60%, 25%, and 10% of the embryos died 1, 2, and 3 days after the treatment, respectively, compared with the control groups in which <14% of the embryos died (Table 1). This effect on the control groups could be associated with manipulation, as reported in our previous studies [13,15,16]. Thus, TP extract decreases significantly the survival probability of the exposed embryos in comparison with their matched controls (Figure 1); meanwhile, it is important to highlight that 104 of 110 embryos died before 7 days ( $p < 0.001$ ) of incubation. Surviving embryos were autopsied at 6–7 days of incubation. On the other hand, we noted that TP extract inhibits angiogenesis of CAM by approximately 40% and in the embryo, especially the brain (data not shown).

Based on our earlier studies related to toxicity during embryogenesis [15,16], we further examined the expression of ATF-3, BCL-2, CASPAS-8, inhibin subunit beta A (INHBA), VEGF-C, and Cadherin-6 type-2 genes in heart and brain tissues from TP-exposed embryos in comparison with unexposed ones (controls) by qRT-PCR. We found that ATF-3,

**TABLE 1.** The number of chicken embryos used in this investigation

Embryo	Number of cases	Number of embryos died before 6 days of incubation (%)
TP-exposed	110	104 (94.54)
Control	30	4 (13.33)

We note that approximately 95% of TP-exposed embryos die 3 days after exposure. The embryos were exposed to TP plant extract at 3 days of incubation as described in the materials and methods section. TP: *Teucrium polium*



**FIGURE 1.** Survival analysis of *Teucrium polium* (TP)-exposed embryos and their matched controls. It is clear that TP significantly decreases the survival probability of exposed embryos in comparison with their controls ( $p < 0.001$ ).

BCL-2, INHB-A, and VEGF-C genes are downregulated, but CASPAS-8 is upregulated in TP embryonic tissues in comparison with tissues from control embryos (Figure 2). Meanwhile, we noted that Cadherin-6 type-2 gene is overexpressed in heart tissues from TP-exposed embryos; in contrast, it is downregulated in their brain tissues. In this regard, it is important to emphasize that these genes are essential regulators of cell proliferation, apoptosis, survival, angiogenesis, and migration, which are critical events during embryogenesis.

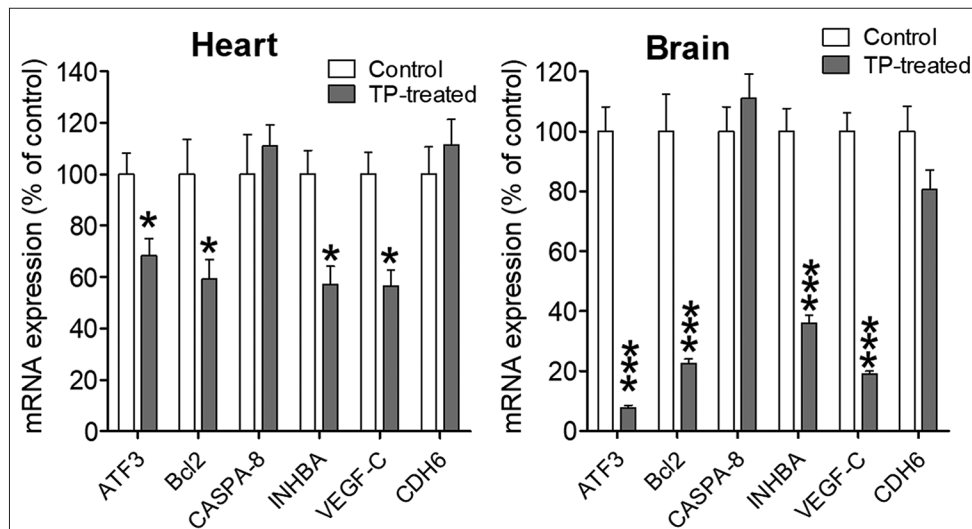
## DISCUSSION

In this study, we investigated, for the 1<sup>st</sup> time, the effect of TP extract during embryogenesis. The outcome of TP extract on women's health is not well understood, especially during pregnancy. However, a few – existing data regarding the effect of TP extract on human health indicate that TP could be harmful during the early stage of normal fetal development, especially since it can induce hepatotoxicity in humans as well as in animal models [11,12,17-19]. This is particularly important since pregnant women, especially in the Mediterranean region, might consume this medicinal plant without being aware of its adverse effects on their pregnancy. On the other hand, recently, Al-Tikriti et al. [4] reported that TP extract downregulates androgen receptors, decrease testosterone levels and sperm count, and, therefore, reduce the general fertility index in rats. Thus, it is evident that TP extract can affect the normal development of the embryo. Accordingly, we reasoned that using the chicken embryo model would be a suitable approach to identify the impact of TP extract during embryogenesis. Thus, we herein report that TP extract can cause a dramatic toxic effect on the embryo at 3 days of incubation, since ~95% of our TP-exposed embryos die after 3 days

of treatment; therefore, it is clear that TP extract severely affects the survival probability of the exposed embryos in comparison with their controls. These data are consistent with several investigations related to the toxic effect of TP extract in animal models [17-19]. Moreover, our study revealed that TP extract could harm the chicken embryo and inhibit angiogenesis of CAM by ~40% after 2–4 days of exposure. This was accompanied by the induction of significant necrosis in different organs such as the liver and other organs (data not shown), which could consequently provoke a high rate of mortality in TP-exposed embryos.

Moreover, our study points out that TP extract can deregulate several key controller genes of cell proliferation, apoptosis, survival, angiogenesis, and migration, which are critical events during embryogenesis, such as of ATF-3, BCL-2, CASPAS-8, INHB-A, VEGF-C, and Cadherin-6 type-2 [15,16]. More specifically, we herein demonstrate that TP plant extract provokes an upregulation of ATF-3, BCL-2, INHB-A, and VEGF-C genes in heart and brain tissues. These data are consistent with our previous work vis-à-vis the outcome of water-pipe smoking (WPS) at the early stage of the normal development of the embryo [15], where we have demonstrated that these genes are important targets of the toxicity incited by WPS. Meanwhile, we report that TP plant extract induces an overexpression of CASPAS-8 in the heart and brain tissues in exposed embryos in comparison with their matched control. It is well documented that CASPAS-8 gene is an important key regulator of cell death and apoptosis [20]. Finally, our data indicate that Cadherin-6 type-2 is overexpressed in heart tissues and downregulated in brain tissues from TP-exposed embryos; this is also in accordance with our previous work on the cytotoxic and genotoxic effect of single-walled carbon nanotubes and WPS on embryogenesis [15,16]. In this context, it is important to highlight that the opposite effect of TP plant extract on Cadherin-6 type-2 gene in heart and brain tissues can be explained as a tissue specificity of gene expression, as demonstrated by several previous reports [21,22].

In conclusion, we herein report that TP extract provokes embryonic death at the early stage of fetal development, which could be driven by the inhibition of angiogenesis, inciting cell apoptosis, and/or disturbing cell migration through the deregulation of ATF-3, BCL-2, CASPAS-8, INHB-A, VEGF-C, and Cadherin-6 type-2, and possibly other genes involved in normal embryonic development. Thus, this study provides strong evidence, for the 1<sup>st</sup> time, that TP extract can harm the normal development of the embryo at the early stage. Therefore, data of this study suggest that TP plant can have very toxic effects on women's health, especially at the early stage of pregnancy. Accordingly, it is important to alert expectant women to avoid the use of this medicinal plant during pregnancy.



**FIGURE 2.** Quantitative real-time polymerase chain reaction analysis of activation transcription factor-3 (ATF-3), B-cell lymphoma-2 (BCL-2), caspase-8 (CASPAS-8), inhibin subunit beta A (INHBA), vascular endothelial growth factor (VEGF)-C, and Cadherin-6 type-2 genes in heart and brain tissues from *Teucrium polium* (TP)-exposed embryos and their matched controls at 6 days of incubation. We note that TP plant extract induces a downregulation of ATF-3, BCL-2, INHBA, and VEGF-C and an upregulation of CASPAS-8 in three samples (embryos) in comparison with control tissues. In parallel, we observe that Cadherin-6 type-2 gene is overexpressed in heart tissues from TP-exposed embryos; in contrast, it is downregulated in their brain tissues, which could be explained as a tissue specificity of gene expression during embryogenesis [21,22]. Data of this analysis are presented as means  $\pm$  SEM. (\* $p < 0.05$ ; \*\*\* $p < 0.001$ ; TP treated compared with control (non-treated) groups.

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## DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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