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Reproductive safety assessment of *Thymus vulgaris* L. extract and quantification of thymol sulfate in pregnant rats and fetuses using a validated LC/MS method of analysis

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ABSTRACT

The current study aimed to investigate the safety of ingesting standardized thyme extract (*Thymus vulgaris* L.) during pregnancy in rats. For this purpose, postmortem examination and thymol sulfate (TS) concentration in maternal and fetal effluent extracts were determined. TS concentrations were analyzed by developing and validating a new liquid chromatography–mass spectrophotometer method for quantification. Three dose levels of 1-, 10-, and 20-fold (x) of the recommended dose of standardized thyme extract in parallel to thymol were administered to evaluate the development of rat fetuses from the 5th to the 19th day of gestation. Blood samples from pregnant rats and fetal tissue samples were analyzed to detect the presence of TS that is a major thymol metabolite. The average concentration of TS detected in pregnant rats' plasma-administered thyme extract was 648 ± 106 ng/ml at 1x dose compared to $3,429 \pm 865$ ng/ml for its equivalent amount of thymol compound. Quantities of TS in the dams and fetal tissue were found to increase in a dose-dependent manner. The administration of the 1x and 10x doses of the extract resulted in a significant increase in the fetal and placental weights. However, 20x dose of both thyme extract and thymol compound exhibited an increase in the number of adsorbing sites in the uterus and reduced birth weights compared to control. In conclusion, TS was capable of crossing the placental barrier in rats and increased the resorption index when high and repeated doses of thyme extract were administered. The current investigation highlights the expected danger of exceeding the therapeutic dose when consuming thyme remedies during pregnancy.

INTRODUCTION

Most studies on herbal medicinal products are directed toward investigating their possible pharmacological effects and active constituents rather than focusing on their toxicity to the human body, especially during pregnancy. However, the use of most herbal remedies during pregnancy is cautioned, not only to pregnant women but to their fetuses that might also be at risk of toxicity following its consumption (Louik *et al.*, 2010). Common

thyme or garden thyme is a herb of *Thymus vulgaris* L. or *Thymus zygis* L. that belongs to the Labiatae or also known Lamiaceae family (World Health Organization, 1999). Despite the diversity in thyme species, *T. vulgaris* L. is the most commonly used in therapeutic dosage forms (Hosseinzadeh *et al.*, 2015). Thymol (2-isopropyl-5-methylphenol), on the other hand, is a colorless crystalline substance that gives thyme its strong flavor and pleasant aromatic odor. Thyme herb extract (TE) and thyme oil are traditionally used to treat symptoms of bronchitis and whooping cough (Basch *et al.*, 2004; Gavliakova *et al.*, 2013).

Since thyme is usually consumed in considerable amounts as food, known as Za'atar in Arabic, it is described as "generally recognized as safe" (Basch *et al.*, 2004). However, a study in 1980 showed that thymol had produced teratogenic

effects in the developing chicken embryo (Verrett *et al.*, 1980). Additionally, another study confirmed morphological toxic alterations in the histology of liver and thyroid tissues of mice exposed to 2 mg/ml of thymol (Leonardo *et al.*, 2017). Data concerning thyme ingestion during pregnancy needs investigation on its effects in relevant endpoints of prenatal exposure on pregnant test animals and on the developing organism; this as recommended by the Organization for Economic Co-operation and Development guidelines on prenatal developmental toxicity study, which includes the assessment of maternal effects as well as death, structural abnormalities, or altered growth in the fetus (OECD/OCDE, 2018). In addition, determining the maternal/fetal effluent defined by the placental crossing of suspected chemical or metabolite is of major concern. Developing suitable and sensitive analytical methods to carry out quantitative analysis for active natural compounds and their metabolites is considered a key means toward assessing *in vivo* toxicokinetic and safety investigations in herbal medicine (Sharma and Sarkar, 2013; Woo *et al.*, 2012). The inherent complexity and variability of herbal extracts have been a significant challenge for separation and detection methods during the analysis of herbal medicine (Marston and Hostettmann, 2009). Additionally, in pharmacokinetic and pharmacodynamic studies, qualification and quantification of metabolites of natural products in complex biological matrixes also require advanced analytical methods with high sensitivity and selectivity (Gray *et al.*, 2010). In this context, tandem mass spectrometry has become a routine analytical technique in clinical practice for quantitative analysis of natural products (Wu *et al.*, 2013). Prior to any liquid chromatography–mass spectrophotometer (LC/MS) analysis, extraction of the desired chemical constituents from the herbal mixtures or biological samples is required to get sample aliquots free of interferences and may also require concentrating the analyte for improved detection or better separation (Wu *et al.*, 2013). Such analytical techniques should be developed and validated prior to implementation.

The current research aimed to evaluate the preclinical safety of thyme extract and its metabolite, namely thymol sulfate (TS), during pregnancy in both pregnant rats and their fetuses initially through observing its effects on the mothers and uterine content. In addition, quantification of TS was carried out utilizing a developed and valid LC/MS method in order to provide insight into the quantities of the metabolite that crossed the placenta reaching the fetus. Such an approach intends to reveal the concentrations that possibly alter the placental functions and therefore could affect the growth of fetuses. An objective of the study is to quantify thymol sulfate (a major thymol metabolite) in samples from mother and fetus through LC/MS.

MATERIALS AND METHODS

Materials

Thymi herba ethanolic extract (TE) (*T. vulgaris* L., 80% native) containing 0.65% of thymol monoterpene was obtained from Martin Bauer Co. (Germany). Pure thymol (T) with more than 99.5% purity was obtained from Sigma Aldrich (United States). TS potassium salt was obtained from Toronto Research Chemicals (Canada), while the internal standards (IS), namely hydrochlorothiazide (HCT) along with 5% formic acid (FA), ethyl

acetate (EA), ammonium chloride, and methanol (MeOH), were all purchased from Thermo Fisher (United States). All herbal materials were kept in dark amber containers at room temperature.

In vivo dose preparation

The first dose level of TE, i.e., the 1x dose, was calculated as 93.75 mg/kg, which represents the human recommended daily dose (RDD) and prepared in distilled water (Basch *et al.*, 2006). The RDD selection was based on prescribing up to 30 mg/day of cough syrup (15 g/100 ml) to pregnant women by healthcare practitioners. On the other hand, since each 100 mg of the standardized extract contains 0.65 mg of thymol (T) according to the source of the extract supplier, the 1x dose of T was calculated as 0.60 mg/kg and prepared in a propylene glycol solution [polyethylene glycol (PG); BBC Chemicals, Torre Boldone, Italy]. All formulations were freshly prepared on the day of administration to ensure consistency and stability of the preparation.

In vivo prenatal development parallel study design

Animal handling

Adult male and female healthy Wister rats with an average age range of 10–12 weeks were housed in the Animal House of the Applied Science Private University (Amman, Jordan) in solid-bottom polycarbonate cages. The rats were accurately weighed and accommodated in an air-conditioned environment (20°C–25°C), having a relative humidity of 30%–70%, and exposed to a 12-hours daily photoperiod cycle. Animals were offered a conventional laboratory diet and clean tap water *ad libitum*. Rats were examined daily for external signs of toxicity (diarrhea, weight loss, or bleeding) or behavioral abnormalities. All animal procedures and the study protocol were revised and approved by the ethical committee of the University of Petra, Amman, Jordan (ethical approval number: 2016/2017-66).

Mating and pregnancy induction

The polygamous design was adapted to induce pregnancy in rats where each female rat was caged with 2–3 males and monitored for any copulation behavior considering day 0 of gestation as the day on which formation of a vaginal plug was observed (OECD/OCDE, 2018). All pregnant rats were daily observed throughout the period of gestation for mortality, morbidity, general appearance, and abnormal behavioral changes.

Study design

Rats received daily oral doses of corresponding treatments using an oral gavage needle from the 5th day of gestation until day 19th day, which is defined as the period of structural development of the embryonic stage for rats (OECD/OCDE, 2018; Wang *et al.*, 2014). The pregnant rats (*n* = 40) were marked and randomized into different groups, where rats in groups 1, 2, and 3 were orally administered with 1, 10, and 20x doses of TE that are equivalent to 93.75, 937.5, and 1,875 mg/kg, respectively, along with group 4 receiving distilled water (5 ml/kg) and serving as a placebo control for TE-treated groups. In addition, rats randomized in groups 5, 6, and 7 were administered with 1, 10, and 20x dose of T (equivalent to 0.6, 6, and 12 mg/kg, resp.) along with group 8 receiving PG solution as a placebo control for T-treated groups (Fig. 1). Rats

were weighed weekly from gestation day 0 to the day of humane termination.

Sample collection

Caesarean procedure

On the 19th day, dams were sacrificed by cervical dislocation and blood samples were collected from rats by cardiac puncture and drawn into Ethylenediaminetetraacetic acid (EDTA) microtainer tubes. The tubes were directly centrifuged for 5 minutes at 4,000 rpm and plasma was separated into labeled tubes and stored at −60°C until requested for analysis. Rats were then immediately prepared for a caesarean section and their uterus horns were removed and weighed. All placentas were isolated and grossly examined for changes in color or presence of lesions. Uteri were then carefully removed and accurately weighed. The number of live, dead, or resorbed fetuses was recorded and all fetuses were inspected for external malformations or teratogenic signs in the head, cleft palate, limbs, or tail (OECD/OCDE, 2018).

Fetuses and placentas were weighed for each litter. Thereafter, fetuses of each group were then pooled and homogenized using a polytron tissue homogenizer and then centrifuged for 5 minutes at 1,000 rpm. The supernatant of each pooled sample was then separated and properly labeled. Maternal and fetal extracted samples were preserved at −60°C until LC/MS analysis of TS.

Data presentation and analysis

Relative uterus weight was calculated as organ to body ratio (using the terminal body weight) using the following equation:

Relative uterus weight = absolute weight of uterus / weight of rat in the day of sacrefice

In addition, the following equation was used to calculate resorption index:

Resorption index = total number of resorption sites / total number of implantation sites × 100

All data points are represented as mean ± standard error of the mean. The statistical significance of difference in mean of a normally distributed variable was assessed using analysis of variance, followed by Tukey's post-hoc test carried out using International Business Machines Corporation (IBM) statistical package for social science program version 25 (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was considered to be statistically significant.

Chromatographic conditions and TS method analysis and validation

Instrumentation and chromatographic condition

An high-performance liquid chromatography (HPLC) from Agilent Technologies, CA (model Agilent 1,200), was arranged, which comprised an online vacuum degasser, a solvent delivery system pump, an autosampler, and a thermostat column compartment combined with an MS detector from SCIEX Company (model API 4,000). The used column was ACE-C8 column (50 × 4.6 mm i.d. 5μ) obtained from Thermo Electron Corporation. The mobile phase was composed of 30% 0.5 mM ammonium chloride: 70% MeOH ratio, while the flow rate was adjusted to 0.65 ml/minutes. The injection volume was 10 μl and the temperature of the autosampler was adjusted to 25°C in addition to other chromatographic conditions summarized in Table 1.

Preparation of internal standard solution

A 1 mg/ml stock solution of the internal standard was prepared by dissolving 10 mg of HCT in 10 ml methanol and then further diluted to produce 2 μg/ml HCT solution.

Preparation of the standard curve and QC samples of TS

The lower limit of quantification (LLOQ) was set at 40 ng/ml. Eight concentrations ranging from 40–3,000 ng/ml were prepared by spiking rat plasma samples with TS to construct its standard curve. A representative chromatogram and a standard curve are shown in Figure 2.

Liquid–liquid extraction method

Plasma samples (0.2 ml) were transferred into Eppendorf tubes and spiked with 50 μl of the IS solution and 75 μl of 5% FA and vortexed for 1 minute. EA (4 ml) was then added and the tubes were vortexed for 5 minutes and centrifuged for 4 minutes at 4,400 rpm. The samples were frozen for 30 minutes at −40°C and then the supernatant organic layer was separated and evaporated
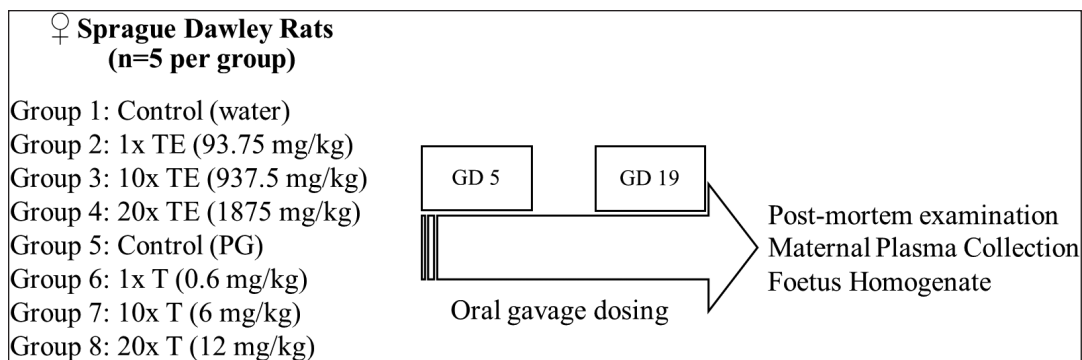

Figure 1. Schematic design of prenatal toxicity experiment on thyme extract (TE) and thymol (T) in Sprague Dawley rats. PG = polyethylene glycol; GD = gestation day.

Table 1. LC/MS chromatographic conditions applied for the detection of TS.

	Flow rate				0.65 ml/minutes		
HPLC	Autosampler injection volume				10 µl		
	Autosampler temperature				25°C		
	Column oven temperature				30°C		
Chromatography	Mobile phase				30% of 5 mM ammonium chloride : 70% Methanol		
	Column type				ACE C8 column (50 × 4.6 mm i.d. 5 µm)		
	Expected retention time:				TS: 0.91 minutes		
					HCT (IS): 0.79 minutes		
MRM detection		Q1 mass	Q2 mass	Dwell time	DP	CE	EP
	TS	228.842	148.6	150.0	−45	−30	−15
	HCT (IS)	295.800	268.5	150.0	−45	−28	−10
Source/gas	CUR	CAD	iS	TEM	GS1		GS2
	25	8	−4500	600	55		50

HPLC = high-performance liquid chromatography; MRM = multiple reaction monitoring; TS = thymol sulfate; HCT = hydrochlorothiazide; Q(n) = mass analyzer stage number; DP = declustering potential; CE = collision energy; EP = entrance potential; CUR = curtain gas; CAD = collision gas; IS = internal standard; i.d. = internal diameter; iS = ion spray voltage; TEM = temperature of ion source; GS1 = nebulizing gas; GS2 = drying gas.
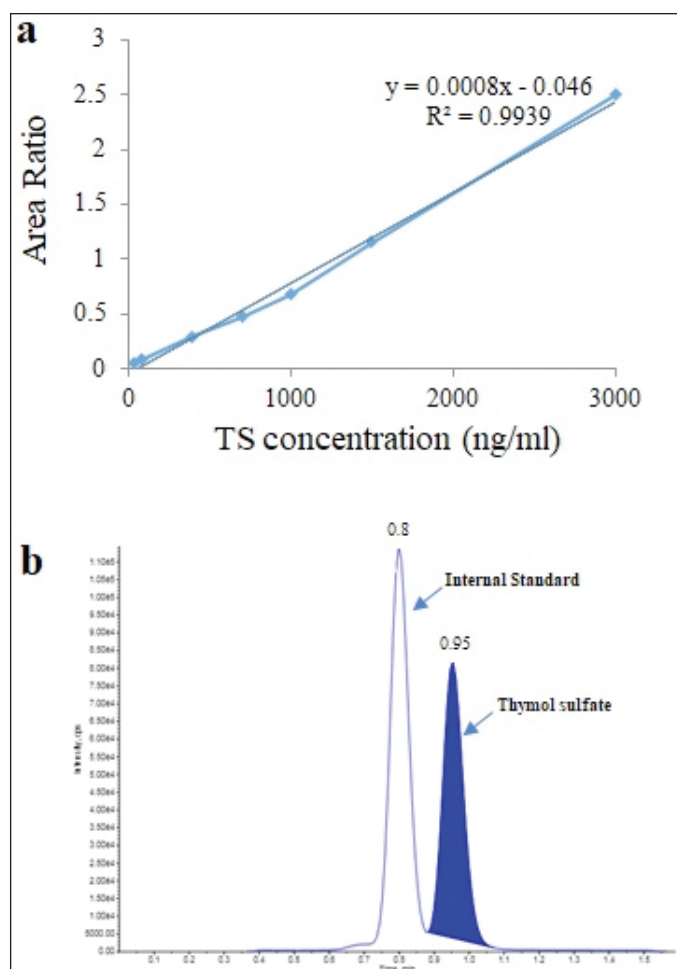
Figure 2. (A) Calibration curve of linearity of intraday validation; (B) LC/MS chromatogram of TS and internal standard. TS = thymol sulfate.

at 40°C in a water bath under airflow for 30 minutes. The obtained residue was reconstituted with 250 µl of 0.5 mM ammonium chloride:MeOH (30:70) and vortexed for 1 minute to ensure complete dissolution and readiness for analysis.

Validation of TS method of analysis

The validation was carried out based on the European Medicines Agency (EMA) guidelines (EMA, 2012). The validation assessed included specificity, linearity, intraprecision, and accuracy.

Specificity

The selectivity was evaluated by analyzing 12 spiked matrix samples at the LLOQ level from six different rat plasma sources.

Linearity and LLOQ

The linearity was measured by assaying calibration curves in rat plasma and was plotted as the peak area ratio (analyte peak area/IS peak area) versus nominal concentrations. Curves were fitted by least-squares linear regression and the acceptable criterion was that correlation coefficient (r^2) was ≥ 0.99. The concentrations of calibration standards defined a lowest concentration as LLOQ compared with nominal concentrations. The accuracy was within ± 20% and the precision was ≤ 20%.

Accuracy and precision

Accuracy and precision evaluations were determined by the analysis of six replicate Quality Control (QC) samples at four levels. The relative error (RE%) was used to assess accuracy, while the calculation of the coefficient of variation (CV%) was used to assess precision. The acceptance criteria of intraday precision for LLOQ and three QC levels were less than 15% and the accuracy was within ± 15% (Table 2).

RESULTS

Method validation

The intraday precision and accuracy values of QC and LLOQ samples are presented in Table 2. The intra-assay mean precision was less than 7.01% and the accuracy range was between 87.32 and 113.70%. The calibration curve of concentration of TS over the range of 40–3,000 ng/ml was established and linearity was obtained with $r^2 \geq 0.993$. The typical equation of the TS calibration curve was $y = 0.0008x – 0.046$, where y represents the ratio of the peak area of the analyte to that of IS and x represents the plasma concentration (Fig. 1). The LLOQ of TS in rat plasma was 40 ng/ml; the sensitivity was enough to determine the plasma concentration and investigate TS concentration in both rat maternal and fetal tissues. The mean intra-assay precision (CV%) at LLOQ concentration was 2.31%, with a calculated RE of 12.65%.

In-life observation of dams following TE and T administration

Treatment with TE and T in the three selected dose levels was generally well tolerated showing no external signs of toxicity. An increase in relative weights of rats was recorded at gestation day 0 and the termination day was considered clinically nonsignificant for all groups. No mortality cases were recorded in dams in all groups.

Postmortem examination and gross observations of uterine, placenta, and fetuses

No signs of abortion or premature delivery prior to scheduled caesarean sectioning were noted in dams. Uteri of each dam submitted to macroscopic examination reported no abnormalities. The average relative weights of uteri and placentas were recorded and no significant difference ($p > 0.05$) was found in the weight of uterus in all treated groups in comparison to control (Table 4). However, induction in placenta masses of TE-treated groups was considered significant (p value > 0.01), despite neglected changes in placenta weights of T-treated groups, in comparison to corresponding control groups. Uterine content was examined for embryonic/fetal deaths and viable live neonates, recording one dead fetus in a 20x TE-treated mother.

The number and distribution of intrauterine implantations were recorded for each treated group and the resorption index was calculated (Table 4). When compared to corresponding control, implantation sites were found to be increased in T-treated groups, despite the dosing concentration. On the contrary, a low number of implanted fetuses was evident in rats of 20x TE group. The average fetal count in dams of other dosing groups including TE- and T-treated was insignificant when compared to that of the control ($p < 0.05$). Total resorption in fetuses of pregnant rats was found to be the highest in dams treated with 20x TE, specifically, 21.42% higher when compared to the control group.

Examination of neonates was made for alterations and malformation or anomalies where each neonate was grossly examined for external defects, including the presence or absence of the head, cleft palate, limbs, and tail. The general conditions of neonates were all favorable, and no anomalies were clear to sight. One mummified fetus among the dams administered 20x TE showed signs of shrinkage (Fig. 3). The average weights of fetuses were significantly increased in all TE-treated groups ($p > 0.01$). On the other hand, among fetuses of dams treated with 1x T, low birth weights were observed (Table 4). The higher doses of T, however, did not show any significant changes in body weights compared to the control group.

Plasma concentration of TS in rat dams and fetuses

The plasma concentrations of TS in dams and fetuses for each dosing group are shown in Figure 4. Trend-like proportional increase in TS concentration was observed in maternal and fetal samples. Maternal concentrations were found to be statistically significant between the 1x TE dose and the other groups ($p < 0.01$). Furthermore, the difference in TS fetal concentration between 1x TE and T dose levels was found to be significant ($p < 0.05$). A dose-dependent increase in TS concentration in fetal tissue was noted in both treatments, where the difference in TS concentration

Table 2. Accuracy and precision for intraday QC samples.

Sample name	Concentration (ng/ml)		RE%	CV%
	Nominal	Calculated		
LLOQ	40	45.06	12.65	2.31
QC$_{Low}$	120	127.78	6.49	7.01
QC$_{Medium}$	1,500	1,603.93	6.93	3.91
QC$_{High}$	2,400	2,731.81	13.83	1.04

SD = standard deviation; RE = accuracy (% mean relative error); CV = precision (% coefficient of variation).

Table 3. Accuracy percentage-based intraday-measured TS concentrations of standard points.

Concentration (ng/ml)		TS peak area	IS peak area	Area ratio	RE%
Nominal	Calculated				
40	43.75	283,921	6,042,777	0.04	9.40
80	90.96	489,786	5,920,521	0.08	13.70
400	377.12	1,843,609	6,157,355	0.29	−5.72
700	611.22	2,814,388	5,904,200	0.47	−12.68
1,000	876.40	4,177,082	6,165,617	0.67	−12.36
1,500	1,509.93	6,847,386	5,919,307	1.15	0.63
3,000	3,245.26	15,211,495	6,083,993	2.50	8.17

TS = thymol sulfate; IS = internal standard; RE = accuracy (% mean relative error); $R^2 > 0.993$; slope = 0.0008; intercept = −0.046; correlation = 0.996.
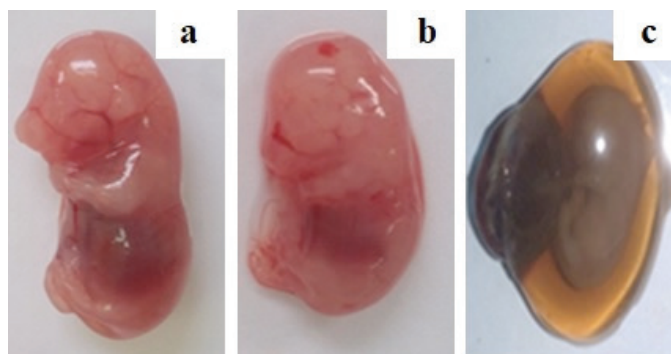

Figure 3. Representative rat maternal fetuses. (a) Control; (b) low birth weight fetus 1x T; (c) mummified rat maternal fetus treated with 20x TE. TE = thyme extract; T = thymol compound.

Table 4. Postmortem examination and gross observations of rat uterine, placenta, and fetuses.

	Control (DW)	1x TE	10x TE	20x TE	Control (PG)	1x T	10x T	20x T
Relative uterus weight (g)	0.043 ± 0.003	0.051 ± 0.008	0.047 ± 0.004	0.036 ± 0.01	0.030 ± 0.007	0.030 ± 0.01	0.030 ± 0.01	0.031 ± 0.01
Relative placenta weight (g)	0.38 ± 0.06	0.49 ± 0.01**	0.49 ± 0.04**	0.57 ± 0.03***	0.43 ± 0.06	0.39 ± 0.05	0.52 ± 0.2	0.42 ± 0.12
Total early resorbed sites	1	1	1	6	0	1	1	2
Total late resorbed sites	0	0	0	1	0	0	1	0
Mean live neonates	8.6 ± 2.33	9.0 ± 2.54	9.4 ± 1.14	4.2 ± 2.5*	6.5 ± 1.5	7.0 ± 4.06	6.14 ± 3.71	6.33 ± 3.39
Neonatal weight (g)	1.37 ± 0.33	1.99 ± 0.07***	1.85 ± 0.18**	1.92 ± 0.12**	1.26 ± 0.06	0.74 ± 0.02*	1.24 ± 0.15	1.05 ± 0.36

DW = distilled water; TE = thyme extract; PG = polyethylene glycol; T = thymol compound.
*p value > 0.05; **p value > 0.01; ***p value > 0.001 using *t*-test, in comparison to corresponding control using *t*-test ($n = 5$).

between the low and the high doses was reported to be significant ($p < 0.05$; Fig. 4).

DISCUSSION

The current study aimed to investigate the safety of ingesting standardized thyme extract during pregnancy through studying it in rats. The influence of TS, a metabolite of thyme, was evident in the fetal fraction which indicates the capacity of TS of crossing the placental barrier. Such an event reflected potential toxicity to the pregnancy and the fetuses accumulated by the incautious use of medical plants. Owing to their therapeutic effects, many medicinal plants are used by pregnant women and are often consumed based on traditional habits or personal experience, and in most cases, information considering the safety of plant use during pregnancy is questioned (Adams *et al.*, 2010; Illamola *et al.*, 2019). In addition, herbal medicines are available over the counter, making them easily accessible and putting pregnant women under risk when self-prescribed. Thyme herb, namely *T. vulgaris* L. or thymol-containing herbs such as *Origanum syriacum* L., are commonly advocated against colds, gastralgia, and anxiety (Illamola *et al.*, 2019). However, the effect of thymol-containing herbs on the developing fetus is still unknown. Given that the American Herbal Products Association (American Herbal Products Association, 1992) states that thyme is contradicted in pregnancy and previous studies have contradictory reports on its safety (Cohen, 2013; Jyoti *et al.*, 2018; Laelago, 2019), studies on the developmental toxicology of thyme are warranted.

The current study developed and validated an LC/MS method for detection and quantification of rat plasma TS, a metabolite of *T. vulgaris* L. extract in fetus and maternal uterine tissues through the placental transmission. This method was found to be robust and reproducible and had a high sensitivity for TS with an LLOQ of 40 ng/ml. Therefore, the plasma was validated over a concentration range of 120–2,400 ng/ml and good linearity was obtained with $r^2 > 0.993$. The mean intra-assay precision (CV%) was less than 7.01% with mean values of accuracy between −12.68% and 13.7%, which revealed that the current method was reliable, accurate, and reproducible. The validated LC/MS method was used to determine quantities of thyme metabolite, namely TS in rat maternal and fetal effluent samples, and provided sufficient and consistent readings. To our knowledge, this is the first study to determine TS in rat plasma through LC/MS and report insights into the transplacental transfer of TS.

This study has demonstrated that exposure to thymol-containing compounds, represented in the study by TE or T administration at concentrations that are equivalent to those
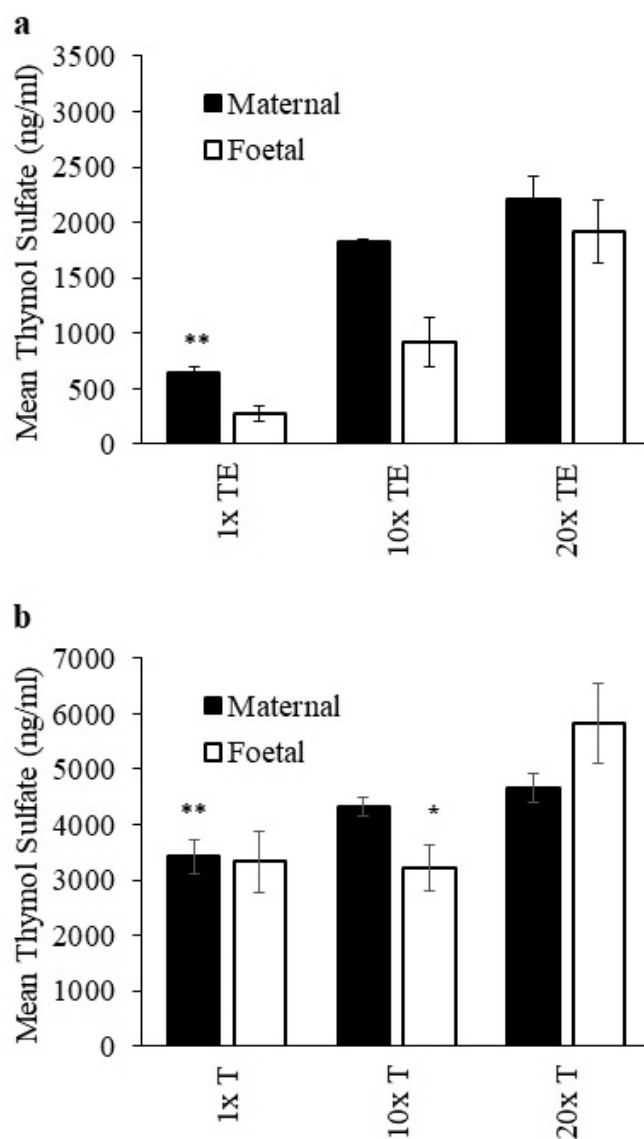
Figure 4. Mean TS concentration in maternal and fetal effluent extracts treated with three dose levels of (a) TE and (b) T. ** = significant in comparison to maternal 20x ($p < 0.01$); * = significant in comparison to fetal 20x T ($p < 0.05$). TE = thyme extract; T = thymol compound.

advocated for the treatment of cough or bronchitis, could result in several changes to fetal development. However, clinicians usually prescribe thyme products to pregnant women at a dose equivalent

to 93.75 mg/kg/day (based on 15 g/100 ml syrups). Therefore, in the current investigation, the dose of 93.75 mg/kg/day of TE was considered as the RDD. Thymol has been reported to be metabolized by CYP450 isoform 2A6 (Dong *et al.*, 2012) and then sulfated by sulfotransferase producing TS, which was reported as the predominant metabolite of thymol in plasma (Kohlert *et al.*, 2002). Thus, TS was selected as a quality marker of TE and T.

Nonetheless, by observing potential adverse effect levels, no alterations on maternal well-being were found nor to weight gain during the dosing period. Although the mean change in placenta weights was not evident in groups treated with thymol, relative placenta weights in thyme extract-treated dams were significantly higher when compared to the corresponding control. It has been previously reported that thyme extract acts as an appetite stimulant; thus, heavier placenta weight could be related to enhanced maternal nutrition accounted to the enhanced appetite after treatment with thyme extract (Dauqan and Abdullah, 2017). This could have impacted placental growth, thereby affecting uteroplacental blood flow and nutrient uptakes by the fetus, all of which play an important role in fetal growth, metabolism, and birth weight (Redmer *et al.*, 2004). However, there was no apparent concentration-related effect as both compounds showed similar relative uterus weights.

The mean number of implantations and litter size per dam did not differ among all groups except for 20x TE group, where the mean live neonate number was reduced to more than a half. In addition, the number of visible early resorptions in 20x TE group was six times higher than the lower doses. The embryonic observations reported no phenotypic anomalies on fetal development among most fetuses. However, the administration of 1,875 mg/kg (20x) TE caused a higher rate of resorption which reached 21.42% and a lower number of fetuses, in addition to one case of intrauterine death found as the early mummified fetus. Such observations were parallel to high TS quantities in 20x TE fetuses in comparison to TS concentrations that reached fetuses in rats treated with lower TE doses. On the other hand, resorption in T-treated groups was twice higher in doses higher than 1x T. Furthermore, low birth weight was evident in fetuses of dams receiving RDD of T. However, growth retardation was evident in pups of 1x T-treated group. It has been suggested that the embryotoxicity represented as resorption may be a result of insufficient hormone concentration and deficient placenta function or may be due to the direct effect of the drug on the embryos (El Gendy *et al.*, 2015). Furthermore, levels of the thyme metabolite TS were considerably high in fetuses of T-treated rats, suggestive of the higher capacity of thymol in crossing the placenta and reaching the fetal effluent. These data combined with the finding of one mummified fetus within the 20x TE group need further in-depth investigation to confirm the raised potential risk of thyme and thymol extract on pregnancy progression. Such an incident needs further investigation in order to inspect the impact of thymol on prenatal development. However, manifestations could be linked to findings of a dose-dependent increase in TS formation in maternal rat plasma. The proportional increase in the transplacental transfer of TS was observed by the increase in TE concentration. As for groups treated with T, concentrations of the metabolite were significantly higher in fetuses of 20x T in comparison to fetuses of lower concentrations.

Presence of TS in fetal tissues reflects its ability to cross the placenta barrier due to its physicochemical properties. Molecules that are relatively lipophilic with low-protein binding and low degree of ionization and have Molecular weight (MW) of less than 600 Da permeate, as is the case with TS, readily cross the placenta layers (Ala–Kokko *et al.*, 1993).

As the fetal kidney develops, more drug metabolites are excreted into the amniotic fluid, leading to the accumulation of drugs within the fetus which is called the ion trapping phenomenon, where more acidic nature of fetal plasma pH is evident compared to maternal plasma. In addition, weak bases and nonionized (lipophilic) molecules can diffuse across the placental barrier and become ionized in the more acidic fetal blood, leading to a net movement from the maternal to fetal systems (Anger *et al.*, 2011).

When thyme-containing remedy is prescribed or consumed, as in most herbal extracts, one should be conscious that constituents other than the active medicinal compound could be present in the extract and could be harmful or toxic. The major phytochemical constituents found in TE include essential oils such as thymol, carvacrol, and *p*-cymene (Dauqan and Abdullah, 2017) and flavonoids including apigenin and luteolin (Javed *et al.*, 2013; Stahl-Biskup and Venskutonis, 2004), in addition to phenolic acids such as rosmarinic acid (Zgórka and Glowniak, 2001). Several studies have investigated such components which can also be found in other plants. In preclinical studies, phenols, for example, were reported to be embryotoxic and fetotoxic, but not teratogenic. Furthermore, phenols generally do not cause developmental defects except at doses that cause maternal toxicity; as for terpenes, they can be chemically classified as alcohols, hydrocarbons, ketones, and epoxides with low cytotoxicity (Mendanha *et al.*, 2013).

CONCLUSION

A selective LC/MS method was successfully developed and validated for quantitative analysis of TS in rat plasma and fetal tissue with high sensitivity and suitable reported LLOQ for TS that reached 40 ng/ml. The current investigation revealed that high doses of thyme extract and its chronic use may alter the placental functions and therefore could affect the growth of fetuses. Based on the results of this study, pregnant females can be advised clinically to use thyme extract only within the recommended doses. Preclinically, further research is still warranted to evaluate the safety of thymol and other thyme constituents on modulating the placental barrier and protein binding. A detailed histological investigation of thyme effects on placental and neonatal vital organs such as the liver and kidneys is also recommended.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

ETHICAL APPROVAL

This study is approved by the ethical committee of the University of Petra, Amman, Jordan (ethical approval number: 2016/2017-66).

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