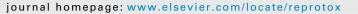
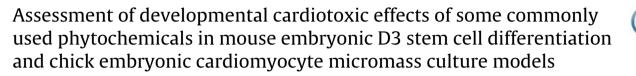
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# Reproductive Toxicology





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# ABSTRACT

Pregnant women often use herbal medicines to alleviate symptoms of pregnancy. The active phytochemicals eugenol (from holy basil) and  $\alpha$ -bisabolol (from chamomile) are recommended to promote calmness and reduce stress. There is evidence that both eugenol and  $\alpha$ -bisabolol possess pro-apoptotic and antiproliferative effects and induce reactive oxygen species. The potential effect was examined by monitoring cardiomyocyte contractile activity (differentiation), cell activity, protein content and ROS production for mouse D3 embryonic stem cell and chick embryonic micromass culture. The results showed that eugenol (0.01–80  $\mu$ M) demonstrated effects on cell activity (both systems) and ROS production (stem cell system only), as well as decreasing the contractile activity and protein content at high concentrations in both systems. Additionally,  $\alpha$ -bisabolol (0.01–80  $\mu$ M) at high concentrations decreased the contractile activity and cell activity and in the stem cell system induced ROS production and decreased protein content. The results suggest only low concentrations should be ingested in pregnancy.

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# 1. Introduction

# 1.1. Overview

Women experience a variety of pregnancy symptoms due to normal physical and biomedical changes, but the range of symptoms and their degree of severity are specific to each individual. This may be due to individual differences in both biological and psychological characteristics. With 1 in 6 pregnancies being unplanned [1], in addition to the expected possible symptoms of pregnancy such as headache, constipation, muscle pain, nausea and vomiting [2], the added stress can have further negative effects for both the mother and unborn child, including low birth weight, pre-eclampsia and pre term birth [3]. For the mother, asthma, coronary heart disease, hypertension, and diabetes can be triggered from the physical stress pregnancy enforces on the body [4]. As with the presence of stress, these conditions not only harm the mother, but the fetus as well [5]. Because of all these possible

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http://dx.doi.org/10.1016/j.reprotox.2016.04.011 0890-6238/© 2016 Elsevier Inc. All rights reserved. health issues triggered by pregnancy, the pregnant woman needs close medical attention and treatment. Whilst the millennia-long history of medicinal use of certain herbs supports the idea that they are safe for a developing fetus the research to support this is very limited, and so physicians tend to not advise their use in pregnancy due to lack of evidence [6]. However there is also a preconception that 'natural' is synonymous with 'safe' [7], resulting in many women choosing herbal medications over traditional options, to cope with common pregnancy symptoms, even those deemed safe by professionals [8–10]. In spite of their increasing popularity, few herbs have been tested for their effects on development of the embryo [11]. Moreover, a literature review into the safety of 4 herbs commonly consumed in pregnancy (ginger, chamomile, peppermint and lemongrass) found that 34% of sources analysed deemed these herbs to be unsafe. More confusingly the information on safety of each herb varied from paper to paper [12]. Further research, by Da Silva Costa et al. in 2012, has also shown, using a database search, the wide range of negative effects from a greater spread of medicinal herbs [13]. For this reason, the investigation of herbal supplements is imperative, not only for evidence of beneficial effects, but primarily to ensure their safety for the developing embryo.





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Holy basil (*Ocimum sanctum*, OS), whilst less known in the Northern hemisphere, is a herb used in traditional medicine in the eastern world. It is credited with a whole range of pharmacological characteristics such as antimicrobial activity, hypoglycemic and hypolipidaemic activity, anti-stress activity, immunomodulation, psycho-pharmacological activity, antioxidant, anti-inflammatory, anticancer activity and radio protective activity [14]. On the other hand, chamomile (*Matricaria recutica*), has been commonly used in the western world for many centuries. The multiple ways to administer chamomile have led it to be used as an anti-inflammatory, antiseptic, antispasmodic, anxiolytic and sedative but also to soothe external rashes such as diaper rash or chicken pox [15]. It is chamomile's effect as an anxiolytic [16] and anti-emetic that promote its use in pregnancy [17].

Concentrating on Holy basil, eugenol is one of the main active ingredients in OS. It has been pharmacologically shown in animals that eugenol is quickly distributed throughout the body and has an LD50 of 1930 mg/kg if orally consumed in rats [18]. It plays a major role in the anti-stress and antioxidant activity of holy basil [19] but is found in multiple plants including those readily added as flavourings to be ingested-cloves, cinnamon, nutmeg, basil (sweet, African and Holy), star anise, dill, vanilla, bay laurel and celery. One study, investigating anti-stress activity of eugenol, has concluded that the mechanism of reducing restrained stress in rats is by its modulatory effect on the hypothalamus, pituitary gland, and adrenal gland association communication, or the HPA axis, which is involved in regulating hormones such as cortisol, and the brain monoaminergic system. This modulatory effect results in a decrease in corticosterone and 5-HT levels in the brain [20]. It has been reported that stress hormones provoke adverse pregnancy symptoms such as headache, fatigue, muscle pains, nausea and vomiting [2]. Therefore, if eugenol can suppress these hormones, theoretically eugenol can be effective in reducing some common pregnancy symptoms.

Similar benefits are claimed for chamomile, and specifically  $\alpha$ -bisabolol, the most abundant molecule in the essential oil. Chamomile as a whole herb is attributed with anti-inflammatory properties, the potential ability to inhibit tumor growth and to reduce the risk of death from coronary heart conditions in certain subjects, as well as the ability to relieve colic/diarrhea especially in infants [17]. Taken as a single ingredient tea it has been used as an anxiolytic and sedative [15], with a randomized placebo-controlled pilot trial concluding it to have mixed and modest effects on both mild to moderate insomnia and mild to moderate generalized anxiety disorder (GAD). However, as with eugenol, toxicity has been reported at high levels. The LD50 was determined to exceed 5 g/kg in rats (greater than that of eugenol) or 14.9–15.6 ml/kg dependant on sex [21].

Both tested phytochemicals - eugenol and bisabolol - share important characteristics. For the expectant mother the reduction in anxiety and stress may have beneficial effects on both the gestation period and for the development of the unborn child. The pro-apoptotic effects of each also suggest them to be protective molecules, however cascade pathways induced by apoptogenic stimuli may result in either the survival or death of the targeted cell with the potential to cause embryonic maldevelopment [22]. In this study, two in vitro systems were used to evaluate these components for a cardiotoxic developmental effect; mouse embryonic D3 stem cell (ESCD3 ) derived cardiomyocytes and chick embryonic cardiomyocyte micromass (MM) culture [23,24]. These two systems represent different growing phases of cardiac development. In ESCD3, the stem cells undergo spontaneous differentiation into contracting cardiomyocytes, similar to the natural process of cardiac development [25], whereas, the cardiomyocytes in MM culture were already differentiated at the time of heart explantation.

#### 1.2. Aim of the study

Our group recently showed [26], that some known herbal components exhibited potential teratogenic effects on the growing chick heart and stem cell derived cardiomyocytes. Therefore, this study was conducted to investigate the possible cardiac embryotoxic potential of two active phytochemicals, eugenol and bisabolol, which might be taken by pregnant women in the early stages of gestation as anti-stress herbal remedies or any other indications. The study was based on two established methods, mouse D3 embryonic stem cells culture [24,23] and chick primary embryonic cardiomyocytes MM system [27]. Cardiomyocyte differentiation, cell activity, protein content and ROS production were monitored for both systems. The ESCD3 system was utilised to investigate the potential role of the test phytochemical in preventing or changing stem cell differentiation to cardiomyocytes while the MM system was used to test their effect with respect to the growing differentiated chick embryonic cardiomyocytes. Accordingly, the test phytochemical might hamper stem cell differentiation but show no effect on the chick primary cardiomyocytes, and vice versa. Cytotoxicity could be indicated whenever the protein content or embryoid body size are lower than control while teratogenicity could be indicated by a decrease in contractile activity, cellular activity or increase in ROS production. Contractile activity can measure cardiomyocyte differentiation, and can be altered by a teratogen without cell activity being even affected.

## 2. Materials and methods

## 2.1. Mouse embryonic D3 stem cells (ESCD3) culture

As our research group previously described the technique [23], ESCD3 were obtained from ATCC, Rockvile, USA. The undifferentiated cells were grown in a T-25 culture flask (Thermo-Scientific, UK) using ESCD3 medium, 500 ml DMEM supplemented with (0.1 mM β-mercaptoethanol, 20% heat inactivated fetal bovine serum (FBS), 50 units ml<sup>-1</sup> penicillin/50  $\mu$ g ml<sup>-1</sup> streptomycin, 2 mM Lglutamine, 1000 U/ml leukaemia inhibitory factor (LIF) and 1% non-essential amino acids (NEAA)) (all reagents Sigma-Aldrich, UK). After the ESCD3 were 80-90% confluent in the T-25 flask, the cells were either passaged or harvested. For experimental purpose, the cells were trypsinised by 2 ml 1x trypsin/EDTA (Sigma-Aldrich, UK) and centrifuged at 900 rpm for 5 min at room temperature (RT); then the cell pellet was resuspended in 5 ml ESCD3 medium without LIF to allow differentiation. A cell suspension of  $4 \times 10^4$  cell/ml was prepared (with or without test chemicals (eugenol and bisabolol, Sigma-Aldrich, UK) [0.01-80 µM]) and 20 µl drops were seeded on the internal side of the lid of a 90 mm Petri dish (Sterilin, UK) and cultured as hanging drops after reverting the lids back to the petri dishes containing 5 ml phosphate buffered saline (PBS) (Sigma-Aldrich, UK) to prevent drying of the drops whilst in the 37 °C/5% CO<sub>2</sub> incubator. The first day of drop plating was recorded as day 0. After 72 h (day 3), the embryoid bodies (EBs) formed within the hanging drops were moved to other petri dishes with 10 ml ESCD3 medium without LIF, maintaining the same environment. The EBs were then transferred after two days into 24 well plates as 1 EB/ml ESCD3 differentiation medium in each well, maintaining the same environment up to day 12. The starting time for differentiation in the well was recorded as day 5 and the differentiation scoring started on day 10 and continued for two more days; 11 and 12.

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