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Safety assessment of homeopathic formulations of *Agnus Castus*, *Pulsatilla Nigricans* and its combination using zebrafish embryos

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Abstract

Homeopathy is a system of alternative medicine conceived in 1796. Despite all the apprehensions attached to it, it is the second largest system of medicine according to the World Health Organisation. Manufacturing of homeopathic medicines require raw materials in the form of different plants, chemicals of animal origin and bacteria, microorganisms and certain disease-causing factors. *Agnus castus*, commonly known as the chaste tree, and *Pulsatilla nigricans* are plants whose parts are used for PCOS, premenstrual syndrome and other conditions. Due to their widespread usage in women of childbearing age, it is important to investigate the possible toxicity and safety level of mother tinctures of both *A. castus* and *P. nigricans*. In this study, zebrafish embryos were used to evaluate the embryo toxicity of mother tinctures of the above-mentioned drugs, since they are well established models used to assess the toxicity of compounds. The embryos were exposed to different concentrations (1, 0.1, 0.01, 0.001%) of the respective mother tinctures. Different parameters like coagulation, somite formation, tail detachment and morphological malformations were recorded till 96 hours of post fertilisation. Results showed that exposure to the mother tincture of *A. castus* did not cause embryonic mortality at all the concentrations used in the study, whereas the mother tincture of *P. nigricans* and the combinations of both the mother tinctures caused mortality at a concentration of 1%, while the other concentrations did not cause embryonic mortality. The concentration at which the toxic effects were observed is much higher than the concentrations used in therapy. Further developmental toxicity studies need to be carried out to validate the use of homeopathic drugs since the availability of toxicity study data is meagre.

Keywords: *Agnus castus*, *Pulsatilla nigricans*, zebrafish embryo, toxicity, mortality, developmental abnormalities

Introduction

Homeopathy, a system of medicine founded by the German physician Samuel Christian Friedrich Hahnemann in 1796, is based on the principle of “like cures like.” Despite longstanding scientific controversy, homeopathy continues to be widely practiced in many regions, including the Indian subcontinent, parts of Latin America, and Western Europe, with increasing adoption in countries such as South Africa and Japan. Its persistence is largely attributed to patient popularity rather than robust scientific validation. Homeopathy remains controversial primarily due to its use of ultramolecular remedies diluted beyond Avogadro’s limit, which has led to criticism and debate. Nevertheless, a body of clinical evidence exists which, although inconclusive, suggests that homeopathy may produce genuine clinical effects. In addition, recent in vitro, animal, and physico-chemical studies have proposed potential mechanisms by which such high dilutions may elicit biological responses, thereby sustaining scientific interest in this field.

According to Lobo *et al.*, homeopathic treatment for female infertility has been reported as a promising alternative therapy in women with infertility, particularly in low-resource settings where access to assisted reproductive technologies is limited ^[1]. In countries such as India, homeopathy is often promoted as a safe, cost-effective option for managing infertility.

However, the scientific validity of these claims remains debated, as many available studies lack rigorous design or sufficient statistical power. Furthermore, the claim that homeopathy addresses both physical and psychological components of infertility remains contentious, as the underlying mechanisms of action are still poorly understood. Cerqueira (2017) reported that *Vitex agnus-castus* is effective in alleviating a range of psychic and somatic symptoms associated with premenstrual syndrome (PMS). Subsequent systematic reviews and clinical trials have supported the use of *Vitex agnus-castus* in the management of PMS, premenstrual dysphoric disorder (PMDD), and other menstrual disorders [2, 3]. *Vitex agnus-castus* influences dopaminergic and hormonal pathways involved in regulation of the female reproductive cycle and has been employed in the treatment of menstrual irregularities, luteal phase defects, infertility, fibrocystic breast disease, menopausal symptoms, and diminished libido [3, 4]. Experimental studies have further demonstrated its effects on reproductive hormone modulation in animal models [5].

The application of *Pulsatilla nigricans* in homeopathy for female reproductive disorders has also been described in the literature, particularly in the context of menstrual irregularities and hormonal imbalance, although robust toxicological evaluations remain limited.

Developmental and reproductive toxicity assessments are essential components of pharmaceutical and chemical safety evaluation. A significant proportion of drug candidates fail during preclinical testing due to adverse developmental or reproductive effects [6, 7]. While traditional in vitro assays using cultured cells provide initial toxicity screening, they often fail to accurately predict in vivo outcomes involving complex processes such as absorption, distribution, metabolism, and elimination. Conventional mammalian models used for developmental toxicity testing are expensive, time-consuming, and labor-intensive [8, 9].

In recent years, increasing emphasis has been placed on alternative models that reduce reliance on mammals for regulatory safety testing. Among these, ray-finned fish (Actinopterygii), particularly zebrafish (*Danio rerio*), have emerged as valuable models due to their genetic conservation, rapid development, and experimental accessibility [10]. Early developmental stages are especially sensitive to toxic insults, yet difficult to assess using traditional mammalian systems. The optical transparency of zebrafish embryos and their external development enable direct visualization of teratogenic and embryotoxic effects [11].

Zebrafish embryos offer several advantages for developmental toxicity assessment: conserved developmental pathways, sensitivity to known mammalian teratogens, suitability for high-throughput screening, and a well-annotated genome allowing mechanistic investigations [12-15]. Several fish-based assays are incorporated into internationally accepted OECD guidelines, including the Fish Acute Toxicity Test (OECD TG 203) and the Fish Embryo Acute Toxicity Test (OECD TG 236) [16, 17].

Materials and Methods

Test Substances

Mother tinctures of *Agnus castus* (Chaste tree) and *Pulsatilla*

nigricans (Wind flower) were obtained from SBL homeopathy and a combination of both was prepared. The tinctures were diluted according to standard homeopathic dilutions to obtain four working concentrations:

- 1% (2X dilution)
- 0.1% (3X dilution)
- 0.01% (4X dilution)
- 0.001% (5X dilution)

Alcohol solutions (0.68%, 0.92%, and 1.6%) corresponding to the highest concentration of alcohol in 1% solutions of *Agnus castus*, *Pulsatilla nigricans* and their combination were used as vehicle controls, while dechlorinated water served as the negative control.

Zebrafish Husbandry and Breeding

Adult zebrafish (*Danio rerio*), (N=20 per group) were maintained in duplicate under standard laboratory conditions in accordance with established guidelines. Breeding was set up at a 2:1 male-to-female ratio, with pairs introduced into spawning tanks before the onset of darkness. Embryos were collected the following morning.

Embryo Collection and Selection

Fertilized eggs were harvested, and only normal, viable embryos at early developmental stages were selected for the experiment. Dead, coagulated, or abnormal embryos were excluded from testing.

Exposure Protocol

Twenty embryos were selected for each concentration in duplicate and exposed to the test substances at the respective concentrations in multi-well plates. Each concentration of the mother tinctures, their combination, and control groups (alcohol and water) were tested in parallel.

Observation and Endpoints

Embryos were observed for a period of 96 Hours Post Fertilization (HPF). Developmental parameters and toxicological endpoints were recorded at 24, 48, 72, and 96 hours. The following parameters were assessed:

- Embryonic development: viability and normal morphology.
- Malformations: coagulated embryos, pericardial EDEMA, yolk sac EDEMA, and curved body axis.
- Cardiac function: heart rate (beats per minute) at 96 hpf. Normal range was considered 140-180 bpm.
- Motility: spontaneous movement of embryos/larvae at 96 hpf.

Data Analysis

Observations were recorded as mean values and compared across treatment and control groups. The No Observed Effect Concentration (NOEC) was determined based on the highest concentration at which no developmental abnormalities or mortality were observed.

Results

Cumulative Embryo Mortality

Table 1: Cumulative percentage mortality of zebrafish embryos exposed to *Agnus castus*, *Pulsatilla nigricans* mother tinctures, and their combination (N=20 embryos per group).

Compound (MT)	Concentration	24 hpf	48 hpf	72 hpf	96 hpf
<i>Agnus castus</i>	0.001% (5X)	0	0	0	0
	0.01% (4X)	0	0	0	0
	0.1% (3X)	0	0	0	0
	1% (2X)	0	0	0	0
<i>Pulsatilla nigricans</i>	0.001% (5X)	0	0	0	0
	0.01% (4X)	0	0	0	0
	0.1% (3X)	0	0	0	0
	1% (2X)	25	100	-	-
Combination (AC + PN)	0.001% (5X)	0	0	0	0
	0.01% (4X)	0	0	0	0
	0.1% (3X)	0	0	0	0
	1% (2X)	100	-	-	-
Compound (vehicle)	Alcohol (0.68%)	0	0	0	0
	Alcohol (0.92%)	0	0	0	0
	Alcohol (1.6%)	0	0	0	10

Malformations

At 1% concentration of *Agnus castus*, embryos exhibited progressive malformations over time. By 72 hpf, 25% of embryos showed Pericardial Edema (PE), while 10% developed a curved body axis (CBA) and no yolk sac edema (YSE) was observed. By 96 hpf, the incidence of abnormalities increased markedly, with 75% of embryos

affected by PE, 35% by YSE, and 75% by CBA. In the 1.6% alcohol control group, substantial malformations were also recorded. At 72 hpf, 65% of embryos displayed PE, 20% exhibited YSE, and 25% showed CBA. These abnormalities further intensified by 96 hpf, where 83% of embryos were affected by PE, 66.6% by YSE, and 83% by CBA.

**Fig 1:** Malformations of embryos

Cardiac Function

At 96 hpf, normal embryos displayed 140-180 bpm. *Agnus castus* 1% embryos had an average heart rate of 121 bpm, while alcohol 1.6% controls averaged 106 bpm, indicating bradycardia.

Motility

Hypomotility was observed in *Agnus castus* 1% and alcohol 1.6% groups, while other groups retained normal motility.

NOEC

The No Observed Effect Concentration for both the mother tinctures and their combination was determined as 0.01%.

Discussion

The zebrafish embryo acute toxicity (FET) test, formalized under OECD Test Guideline 236, is a widely accepted method for evaluating developmental and acute toxicity. This assay monitors lethality and sublethal morphological endpoints such as pericardial edema, yolk sac edema, and body axis curvature up to 96 hours post fertilization and has demonstrated predictive value for vertebrate developmental toxicity [18]. The rapid onset of mortality observed in the *Pulsatilla nigricans* (PN) and combination groups within 24-48 hours suggests severe embryotoxic interference, potentially affecting fundamental developmental processes such as cell

division, cardiogenesis, or energy metabolism. Ethanol, used as a solvent in mother tinctures, is a known developmental toxicant. Zebrafish studies have shown that ethanol concentrations $\geq 1.5\%$ induce mortality, edema, bradycardia, and morphological defects [19]. In the present study, 1.6% ethanol caused limited mortality, indicating that ethanol may contribute to toxicity but does not fully explain the complete lethality observed in PN and combination groups.

Early embryonic mortality in zebrafish has been associated with disruption of essential developmental pathways during cleavage and gastrulation stages [20]. The pronounced lethality observed with PN suggests the presence of bioactive constituents capable of exerting potent embryotoxic effects, potentially enhanced through synergistic interactions in combination treatments.

Pericardial edema is among the most sensitive indicators of cardiotoxicity in zebrafish embryos and has been linked to impaired cardiac development, reduced contractility, and vascular dysfunction [21]. The marked pericardial edema observed with *Agnus castus* at 1% concentration, in the absence of comparable effects in corresponding alcohol controls, suggests a compound-specific cardiotoxic effect.

Yolk sac edema is frequently associated with impaired yolk utilization, hepatic dysfunction, or metabolic disruption during early development [22]. The concurrent presence of yolk sac and pericardial edema observed in high-dose groups

is consistent with systemic developmental stress. Zebrafish heart rate, typically ranging from 120-180 beats per minute depending on developmental stage, is a well-established functional endpoint in developmental toxicity studies [23]. Bradycardia observed in both *Agnus castus* and high-concentration alcohol groups supports a cardiodepressive effect.

Curvature and trunk abnormalities reflect disruption of somitogenesis, notochord development, or neural tube formation and are widely used indicators of teratogenicity in zebrafish models [24]. The presence of these defects at higher concentrations indicates broader developmental perturbations beyond isolated cardiac toxicity.

The No Observed Effect Concentration (NOEC) of 0.01% identified in this study for *Agnus castus*, *Pulsatilla nigricans*, and their combination suggests that lower dilutions are well tolerated during early development.

This study demonstrates that *Agnus castus* and *Pulsatilla nigricans* mother tinctures exhibit distinct safety profiles in zebrafish embryos, with *Agnus castus* showing minimal lethality but *Pulsatilla nigricans* and its combination producing rapid, complete embryo mortality at high concentrations. Sublethal malformations, including pericardial and yolk sac edema, bradycardia, and motility defects, were evident at 1% exposure, while concentrations $\leq 0.1\%$ were well tolerated. The No Observed Effect Concentration (NOEC) was established at 0.01%. These findings highlight the utility of zebrafish embryo assays for preclinical safety screening of homeopathic tinctures and warrant further mechanistic and mammalian studies to validate cardiotoxic and embryotoxic risks.

Future work should focus on identifying the bioactive constituents within *Pulsatilla nigricans* and its combination that drive embryotoxicity, using chemical profiling and mechanistic assays. Validation in mammalian models and target species cell-based systems will be essential to establish translational relevance and refine safe dosage thresholds for clinical use.

Conflict of Interest

Not available

Financial Support

Not available

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