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Safety assessment of a standardized polyphenolic extract of clove buds: Subchronic toxicity and mutagenicity studies

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ABSTRACT

Despite the various reports on the toxicity of clove oil and its major component eugenol, systematic evaluations on the safety of polyphenolic extracts of clove buds have not been reported. Considering the health beneficial pharmacological effects and recent use of clove polyphenols as dietary supplements, the present study investigated the safety of a standardized polyphenolic extract of clove buds (Clovinol), as assessed by oral acute (5 g/kg b.wt. for 14 days) and subchronic (0.25, 0.5 and 1 g/kg b.wt. for 90 days) toxicity studies on Wistar rats and mutagenicity studies employing *Salmonella typhimurium* strains. Administration of Clovinol did not result in any toxicologically significant changes in clinical/behavioural observations, ophthalmic examinations, body weights, organ weights, feed consumption, urinalysis, hematology and clinical biochemistry parameters when compared to the untreated control group of animals, indicating the no observed-adverse-effect level (NOAEL) as 1000 mg/kg b.wt./day; the highest dose tested. Terminal necropsy did not reveal any treatment-related histopathology changes. Clovinol did not show genotoxicity when tested on TA-98, TA-100 and TA-102 with or without metabolic activation; rather exhibited significant antimutagenic potential against the known mutagens, sodium azide, NPD and tobacco as well as against 2-acetamidoflourene, which needed metabolic activation for mutagenicity.

1. Introduction

The development and application of natural medicines and health promoting/guarding botanical agents is of great interest since thousands of years. Spices, a group of aromatic plants widely in use as food flavors and preservatives, constitute an important class of medicinal plants well-practiced in Indian and Chinese traditional systems of medicine. Modern scientific research has unraveled the medicinal secrets of spices and delineated their phytochemicals (phytonutrients) responsible for the health beneficial pharmacological activities [21,13,32]. The dried flower buds of clove (*Syzygium aromaticum* L.), an evergreen tropical plant belonging to the family of Myrtaceae, is a popular kitchen spice possessing both food and medicinal applications. It is a rich source of essential oil [12–16% (v/w)] and phenolic compounds [8–12% (w/w) gallic acid equivalent] comprising hydrolysable tannins, phenolic acids and flavonoids [29]. Clove oil and its major component eugenol [70–85% (w/v)], exhibited several therapeutic effects including antibacterial, antifungal, analgesic, antispasmodic, anticarminative, antiseptic, and insecticidal effects in addition to their flavoring applications in food [6,22]. Major pharmacological activities of clove oil and eugenol include antioxidant, anti-inflammatory, antidiabetic, hypolipidemic, antinociceptive, hepatoprotective, antiviral and anticancer properties [29,6,22]. Recently, the nonvolatile polyphenols in clove buds were also shown to be bioactive and started using as dietary supplements [1,18,20]. Aqueous and alcoholic extracts of clove buds rich in polyphenols, such as gallic acid, ellagic acid, tannins, flavonoids and their glycosides were reported to possess aphrodisiac, hypoglycemic, gastroprotective, anti-inflammatory and antithrombotic effects [1,18,20]. However, no systematic studies on the oral toxicity of clove polyphenols are available to date, except a 28-days repeated dose toxicity analysis [18].

The US Food and Drug Administration (FDA) has approved clove buds, clove oil and oleoresins as generally recognised as safe (GRAS) for use as food additives [38]. While the essential oil is mainly responsible for the characteristic pungency and aromatic flavor, the nonvolatile polyphenols provide bitterness and astringency. Clove

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oil and eugenol were classified as minimum risk pesticides by EPA and products containing them are exempted from the requirements of FIFRA [11]. Various acute and chronic toxicity studies of clove oil have reported an oral LD_{50} of 3597.5 mg/kg and has reported no adverse effects when tested for subchronic toxicity tests, with an NAOEL levels of 900–2000 mg/kg/day [33,40]. Oral LD_{50} of eugenol was reported as 2650–3000 mg/kg b.wt. [33,40]. Moreover, eugenol was shown to be rapidly absorbed, metabolized in the liver and eliminated within 24 h when consumed orally [9].

However, scientific information on the safety assessment of clove buds or their polyphenol extracts exhibiting significant biological activity are rare, except a few studies with respect to testicular function in mice [28] and inhalation toxicity of clove cigarettes [26]. So, there exist a necessity of credible data on the toxicity aspects of bioactive and standardized extracts of clove buds to further exploit their functional applications in food, dietary supplements and medicine. Thus, the present study was aimed at the safety evaluation of a water soluble polyphenol rich extract powder of dried clove buds (*hereinafter named as 'Clovinol'*), containing 41.2% gallic acid equivalent of polyphenols. Clovinol was reported to possess significant *in vivo* antioxidant, anti-inflammatory and gastroprotective activities in alcohol induced ulcerative rat model [18]. It has also been demonstrated to exert significant detoxification potential and cardiac health beneficial effects in human subjects, by significantly reducing the lipid peroxidation and enhancing the endogenous redox enzyme levels [20].

2. Materials and methods

2.1. Preparation and characterization of 'Clovinol'

Dried clove buds were received from a selected farm in Indonesia where clove trees are grown without using any pesticides or chemicals. The samples were identified by an authenticated botanist and a voucher specimen (AK-CLV-011) was deposited at the Herbarium of M/s Akay Flavours & Aromatics Ltd., Cochin, India. Clovinol was prepared by hydro-ethanolic extraction followed by purification and spray drying as reported earlier [18] and found to contain 41.2% gallic acid equivalent (GAE), when quantified by standard Folin–Ciocalteu test [34]. HPLC analysis was carried out on a Shimadzu model LC 20 AT, with M20A photo diode array (PDA) detector (Shimadzu Analytical India Pvt. Ltd., Mumbai, India), fitted with a reverse phase C18 column (250 × 4.6 mm, 3 μm) (Phenomenex, Hyderabad, India). Characterization of polyphenols was achieved by 1290 infinity ultra-performance liquid chromatography (UPLC) system coupled with Agilent 6530 QTOF instrument having a Jet Stream source (Agilent India Pvt. Ltd., Bangalore, India). Ammonium acetate (10 mM) in water (A) and methanol (B) was employed as the mobile phase with a Zorbax Eclipse Plus C18 (3.0 × 100 mm; 1.8 μm) column at 30 °C and 5 μL injection volume.

2.2. Animals

Adult Wistar rats (male and female) weighing 170 ± 20 g were used for the toxicological studies. The animals were procured from Veterinary College, Mannuthy, Kerala, India and were acclimatized for a period of 14 days in ventilated cages and housed at the animal house facility of M/s Amala Cancer Research Centre, Kerala, India, in an air-conditioned room at 22 ± 2 °C and relative humidity 60 ± 5% with 12 h light and dark cycle. All animal experiments were carried out in strict accordance with the ethical norms approved by the Institutional Animal Ethics Committee (IAEC) recognized by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Registration No:149/99/CPCSEA). Animals were provided with pellet diet and water *ad libitum*.

2.3. Toxicity studies

2.3.1. Acute toxicity (14 days) study of 'Clovinol'

Acute toxicity studies were initially performed to verify whether Clovinol produced any toxic effects when supplemented at the maximum recommended concentration. Clovinol was suspended in water and orally administered as a single dose at the limit dose of 5 g/kg b.wt. in a sequential manner. Forty rats were divided into four groups, with each group containing five animals per sex having similar weights (170 ± 20 g). Group I was the control (untreated), and Groups II, III and IV were administered with Clovinol at 1.0, 2.0 and 5.0 g/kg b.wt. respectively. All the animals were observed for mortality, clinical and behavioral signs for the first 10, 30, 60, 120, 240 and 360 min post dose, and thereafter twice daily for mortality and once daily for clinical signs during the study period of 14 days.

2.3.2. Subchronic toxicity (90 days) study of 'Clovinol'

Forty Wistar rats (20 males and 20 females) of average weight between 150–200 g were selected by stratified randomization and then divided into four groups, each consisting of five males and five females of approximately the same body weight. Group I was the untreated control animals, administered with 1 mL of water; Group II, III and IV were orally administered with Clovinol at 0.25, 0.5 and 1.0 g/kg b.wt. respectively for 90 days. Clovinol was suspended in distilled water and orally administered to the animals using an oral feeding needle in such a way that all the animals received same volume of vehicle. The animals were monitored for any type of clinical symptoms, mortality, and adverse reactions during the study period. Body weight, food and water consumption were determined every week for 90 days and expressed for a single cage of five animals. After 90 days, the animals were sacrificed by cervical dislocation under ether anesthesia. Necropsy was performed in the presence of a veterinary doctor and examined visibly for any type of abnormalities. All the organs were separated and individual weight was recorded. The weight of brain, liver, stomach, kidney and spleen were recorded and expressed in relation to the final body weight. The tissue samples were fixed in 10% formalin, and embedded tissues were cut into slices of 2–4 μm and stained with hematoxylin and eosin for histopathological examinations with an optical microscope of 100 × magnifications (Olympus-Magnus trinocular microscope, Tokyo, Japan).

Blood was collected by direct heart puncture method into EDTA coated and non-EDTA vials for analyzing the hematological parameters and serum biochemistry. Red blood cells (RBCs) count, total and differential white blood cells (WBCs) count, platelet levels and hemoglobin (Hb) content were determined using hematology analyzer (Model-Diatron, Wein, Austria). Serum was separated by centrifuging at 5000 rpm for 10 min at −4 °C and was stored in a clean sample bottle at −20 °C for further analysis. The total bilirubin was determined as detailed by the Pearlman method [30]; alkaline phosphatase (ALP) was estimated by *p*-nitrophenyl picolinate (PNNP) hydrolysis; alanine amino transferase (ALT) and aspartate aminotransferase (AST) were estimated using kinetic method kits supplied by M/s Raichem, India, using a Microlab 300 auto-analyzer (Merck, Mumbai, India); albumin was determined by its reaction with bromocresol green, and the total protein concentration was determined by the Biuret method [25]. Kidney function markers, such as creatinine and blood urea, were estimated by Jaffe's kinetic and urease methods respectively [14]. The total cholesterol was estimated by the CHOD–PAP (cholesterol oxidase-phenol + aminophenazone) enzymatic method [10]; triglycerides by the GPO–PAP (glycerol-3-phosphate oxidase—phenol + aminophenazone) method [8] and