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An experimental evaluation of the anti-atherogenic potential of the plant, *Piper betle*, and its active constitutent, eugenol, in rats fed an atherogenic diet

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ABSTRACT

Hypercholesterolemia is a major risk factor for systemic atherosclerosis and subsequent cardiovascular disease. Lipoperoxidation-mediated oxidative damage is believed to contribute strongly to the progression of atherogenesis. In the current investigation, putative anti-atherogenic and antioxidative properties of an ethanolic extract of *Piper betle* and of its active constituent, eugenol, were sought in an experimental animal model of chronic hypercholesterolemia. Atherogenic diet-fed rats that received either *Piper betle* extract orally (500 mg/kg b.wt) or eugenol orally (5 mg/kg b.wt) for 15 days (commencing 30 days after the atherogenic diet had been started) exhibited the following variations in different parameters, when compared to atherogenic diet-fed rats that received only saline: (1) significantly lower mean levels of total cholesterol, triglycerides, low-density lipoprotein cholesterol and very low density lipoprotein cholesterol in both serum and hepatic tissue samples; (2) lower mean serum levels of aspartate amino-transferase, alanine amino-transferase, alkaline phosphatase, lactate dehydrogenase and lipid-metabolizing enzymes (lipoprotein lipase, 3-hydroxy-3-methyl-glutaryl-CoA reductase; (3) significantly lower mean levels of enzymatic antioxidants (catalase, superoxide dismutase, glutathione peroxidase, glutathione-*S*-transferase) and non-enzymatic antioxidants (reduced glutathione, vitamin C and vitamin E) and significantly higher mean levels of malondialdehyde in haemolysate and hepatic tissue samples. Histopathological findings suggested a protective effect of the *Piper betle* extract and a more pronounced protective effect of eugenol on the hepatic and aortic tissues of atherogenic diet-fed (presumed atherosclerotic) rats. These results strongly suggest that the *Piper betle* extract and its active constituent, eugenol, exhibit anti-atherogenic effects which may be due to their anti-oxidative properties.

1. Introduction

Hypercholesterolemia is a major risk factor for the development and progression of atherosclerosis and related cardiovascular diseases [1,2]. A positive correlation between serum cholesterol levels and the risk of cardiovascular disease has been reported [3,4]. Oxidative stress reportedly plays an important role in atherogenesis by stimulating the oxidation of lipids and proteins in the vascular wall and by proliferation and migration of smooth muscle cells to the intima [5–7]. Atherosclerotic lesions and endothelial dysfunction have also been linked to generation of reactive oxygen species (ROS). Interestingly, common cardiovascular risk factors that promote atherosclerosis, such as hypercholesterolemia, diabetes mellitus, hypertension, smoking, age and nitrate intolerance, may also increase the production of ROS [8,9]. A high-cholesterol diet (HCD) is a major environmental contributor to disrupted lipoprotein metabolism, and is associated with an increased prevalence of atherosclerosis. Feeding animals with cholesterol is reported to elevate serum or tissue cholesterol levels, facilitating the study of hypercholesterolemia-related metabolic disturbances [10]. Feeding of a cholesterol-rich diet also induces free radical production, followed by oxidative stress and hypercholesterolemia [11–13]. Since oxidative stress appears to play a significant role in the development of atherosclerosis in the vascular wall through the increased formation of ROS, followed by imbalance in the antioxidant status [14], alleviating the deleterious effects of ROS by enhancing

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antioxidant defence mechanisms might possibly retard atherogenesis and therein ameliorate atherosclerosis.

Piper betel Linn. (Piperaceae) is found to grow widely in the tropical humid climate of South East Asia; its leaves, with a strong pungent and aromatic flavor, are widely- consumed as a mouth-freshner. The extract of *Piper betle* leaves contains several bioactive molecules, such as polyphenols, alkaloids, steroids, saponins and tannins [15] and is reported to exhibit antioxidative [16], antihypercholesterolemic [17], anticancer [18], immunomodulatory [19], antimicrobial [20], anti-fertility [21], antiulcer [22], anti-allergic [23], gastro protective [24], antinociceptive [25], wound healing [26], anti-asthmatic [27], detoxifying, and antimutagenic properties [28]. Eugenol (4-allyl-1-hydroxy-2- methoxybenzene), one of the active constituents of *Piper betle,* is widely used as a natural food-flavouring agent since it is found in *Piper betle,* cinnamon, clove, basil, and nutmeg. Previous studies have demonstrated the antioxidant potential of eugenol, which significantly prevents oxidative tissue damage in different experimental animal models [29–31].

The present investigation sought to determine whether an extract of *Piper betle,* and its active constituent, eugenol, can prevent or retard the progression of atherosclerosis in an experimental model of chronic hypercholesterolemia.

2. Materials and methods

2.1. Chemicals

Eugenol (98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals and reagents were of analytical grade and were obtained from HiMedia Laboratories (Mumbai, India).

2.2. Experimental animals

Animals were maintained per national guidelines and protocols approved by the Institutional Animal Ethical Committee (BDU/IAEC/2014/OE/07/Dt.18.03.2014). Male albino rats of the Wistar strain (150–200 g) were used in this study. The animals were housed in clean polypropylene cages under conditions of controlled temperature (25 ± 2 °C) with a 12/12-h day–night cycle, during which they had free access to food and water *ad libitum.*

The experimental animals were fed an atherogenic diet. The diet was prepared by mixing equal quantities of a commercial feed powder (obtained from Sai Durga Feeds and Foods, Bengaluru, India) and atherogenic constituents. The final form of the atherogenic diet contained 5% cholesterol, 20% sucrose, 20% hydrogenated vegetable oil, 2% sodium cholate, 20% lactose, 0.4% choline chloride and 0.15% thiouracil, mixed with an equal quantity (w/w) of the commercial feed powder.

2.3. Experimental design

Male albino Wistar rats were divided into four main groups, each group comprising five rats:

Group I rats (controls) were fed a normal diet *ad libitum* for 45 days and received saline (200 μl/kg b.wt) orally daily for 15 days from days 31 to 45.

Group II rats (atherogenic diet-fed, saline-treated) were fed an atherogenic diet *ad libitum* for 45 days and received saline (200 μl/kg b.wt) orally daily for 15 days from days 31 to 45.

Group III rats (atherogenic diet-fed, *Piper betle* extract-treated) were fed an atherogenic diet *ad libitum* for 45 days and received the *Piper betle* extract in an aqueous suspension (500 mg/kg b.wt./day) orally daily for 15 days from days 31 to 45.

Group IV rats (atherogenic diet-fed, eugenol-treated) were fed an atherogenic diet *ad libitum* for 45 days and received eugenol dissolved in 0.5% peanut oil (5 mg/kg b.wt./day) orally daily for 15 days from days 31 to 45.

At the end of the experimental period, all the animals were sacrificed by cervical decapitation. From each rat, blood samples were collected and the serum was separated. Samples of hepatic tissue were also excised and stored at −80 °C until analysis.

2.4. Biochemical analysis

2.4.1. Preparation of haemolysate

From each blood sample, serum was separated and haemolysate was prepared following the method of Dodge et al. [32], as modified by Quist [33]. In brief, blood samples were collected in ethylenediaminetetraacetic acid [EDTA]-containing tubes and centrifuged at 2000x g for 15 min at 4 °C. The packed cells were washed with 0.89% saline to remove the buffy coat. An aliquot of packed cells was then washed three times with isotonic solution (0.3 M Tris–HCl buffer pH 7.4). One ml of washed cells was lysed using 9 ml of a hypotonic solution (0.015 M Tris–HCL buffer pH 7.2), following which the lysed cells were centrifuged for 30 min at 15,000*g*. The supernatant (haemolysate) thus obtained was used for the assay of antioxidant enzymes. All the samples were stored at −80 °C until analysis.

2.4.2. Preparation of hepatic tissue samples for analysis

Hepatic tissue (100 mg/ml buffer) was homogenized with 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 12,000*g* for 15 mins and the supernatant thus obtained was used for further analysis. The protein concentration of each fraction was determined by the method of Bradford [34], using crystalline bovine serum albumin (heat shock fraction, >98% purity, units = 40 mg/mL H_2O [obtained from Sigma Chemical Company, St. Louis, MO, USA]) as a standard. The absorbance was read at 595 nm using a UV–vis spectrophotometer (Spekol-1300, Analytik Jena, Munich, Germany).

2.4.3. Determination of lipid profile parameters in serum samples

The mean levels of total cholesterol [TC], triglycerides [TG], low-density lipoprotein [LDL] cholesterol, very low-density lipoprotein [VLDL] cholesterol and high density lipoprotein [HDL] cholesterol in the rat serum samples were determined. Serum total cholesterol (mg/dL) was measured by the cholesterol oxidase (CHOD)-PAP method using a standard kit (Cholesterol DES kit, obtained from Transasia Bio-Medicals Ltd., Mumbai, India). Serum HDL-cholesterol (mg/dL) was measured by a modified polyvinyl sulfonic acid and polyethylene-glycol-methyl ether coupled classic precipitation method [35] using a standard kit (HDL-Direct, obtained from Erba Lachema s.r.o., Brno, CZ). Serum triglycerides (mg/dL) were measured by the glycerol-phosphate oxidase (GPO)—Trinder method, as modified by McGowan et al., [36] using a standard kit (Triglycerides DES kit, obtained from Transasia Bio-Medicals Ltd., Mumbai, India). Readings were taken using an auto-analyser (EM 360, Transasia Biomedicals Ltd, Mumbai, India). The serum levels of LDL cholesterol (mg/dL) and VLDL cholesterol (mg/dL) were calculated by Friedwald's formula [37], while the atherogenic index (AI) was calculated as AI = (total cholesterol − HDL)/HDL. Readings were validated by using a known positive control (Seronorm™ Human High, obtained from SERO AS, Billingstad, Norway); the analytical value was 232 mg/dL (18 units) for total cholesterol, 59 mg/dL (6 units) for HDL-cholesterol, and 449 mg/dL (35 units) for triglycerides.

2.4.3.1. Determination of lipid profile parameters in hepatic tissue samples. The mean levels of total cholesterol [TC], triglycerides