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Hepatoprotective effect of *Aegle marmelos* augmented with piperine co-administration in paracetamol model

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ABSTRACT

The current study explored hepatoprotective effect of *Aegle marmelos* (L.) Corrêa, Rutaceae, leaves extract. Potentiation of *A. marmelos* hepatoprotective effect with piperine co-administration was also explored. Wistar rats were randomly divided into seven groups: (i) normal control, (ii) paracetamol group, (iii) silymarin group, (iv) extract-25 group (25 mg/kg body), (v) extract-50 group: (50 mg/kg), (vi) extract-100 group (100 mg/kg) and (vii) extract-25 + piperine group. Hepatotoxicity was induced by administering paracetamol orally in a dose of 400 mg/kg for seven days. The drugs were administered 30 min prior to paracetamol administration and continued for seven days. Animals were 'sacrificed' at the end of treatment and serum was collected for evaluating alkaline phosphatase, bilirubin, lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase IL-10 and TNF-α levels. Liver homogenates were used for determination of oxidative stress (malondialdehyde, reduced glutathione, superoxide dismutase, catalase, glutathione reductase, GSH-S-transferase, glutathione peroxidase and glucose-6-phosphate dehydrogenase). Serum biochemical markers were significantly higher in paracetamol group as compared to normal control group. Significant increase in oxidative stress parameters and inflammatory mediators was also observed. Treatment with *A. marmelos* curtailed the toxic effects of paracetamol in a dose dependent fashion. 100 mg/kg dose of *A. marmelos* was found to be most hepatoprotective. The results of extract-100 group were comparable to silymarin group. Low dose of *A. marmelos i.e.*, 25 mg/kg was combined with piperine to evaluate potentiation of hepatoprotective effects of *A. marmelos*. Piperine co-administration potentiated the hepatoprotective effects, because the combination group results were comparable to high dose *A. marmelos* group. *A. marmelos* exerts hepatoprotective activity through its antioxidant and anti-inflammatory properties which was enhanced by piperine.

Introduction

Liver plays a major role in various stages of biochemical and physiological activities such as energy and nutrient supply, homeostasis, immunity, detoxification as well as metabolism and storage of nutrients (Singh et al., 2016). Industrial toxins, drugs, free radicals, food additives and alcohol are the risk factors for developing liver diseases (Abirami et al., 2015). Drugs causing hepatotoxicity include PCM, NSAID, statins, isoniazid, and various anti-microbial agents (Verma and Neil, 2009). Treatment of drug induced hepatotoxicity is mainly supportive and discontinuation of offending drug is the first step. Certain hepatotoxic drug specific treatments are also available; for example, liver injury due to valproate is treated with carnitine. Likewise, PCM induced liver injury is treated with *N*-acetylcysteine (Leise et al., 2014). Search for newer efficacious hepatoprotective drug fewer side effects is desirable (Mahmood et al., 2014). Recent research has focused on evaluation of hepatoprotective natural products.

Aegle marmelos (L.) Corrêa, Rutaceae, popularly known as Bael in India, is a tough subtropical tree found all over the sub Himalayan forest. The fruits, roots, leaves, bark and seeds of the tree are reported to have medicinal value (Baliga et al., 2011). *A. marmelos* leaves contain large number of phytochemicals such as eugenol, lupeol, cineol, citronellal, cuminaldehyde, skimmianine, citral, aegeline and marmesinine (Maity et al., 2009). *A. marmelos* leaves have been traditionally used in the treatment of fever, cardiac dysfunction, hepatitis, asthma, diabetes, dyspepsia, seminal weakness, inflammation and febrifuge (Sharma et al., 2011). Earlier reports

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have shown antioxidant and nitric oxide scavenging properties of *A. marmelos* fruit pulp aqueous extract (Kamalakkannan and Stanely, 2003; Jagetia and Baliga, 2004). Preclinical studies of *A. marmelos* whole plant extract and fruit pulp extract showing hepatoprotective effect are also reported. The observed hepatoprotective effects were attributed to enhancement of antioxidants levels and preventing hyper-proliferation in liver (Khan and Sultana, 2009; Rajasekaran et al., 2009). Previous studies with *A. marmelos* mainly explored pharmacological properties of the fruit. Hepatoprotective effect of *A. marmelos* leaves is not much explored.

Piperine is an alkaloid present in fruits of *Piper nigrum*, Piperaceae. Various pharmacological studies have showed diverse pharmacological properties of piperine (Lee et al., 1984). Piperine has been shown to possess anti-epileptic, analgesic and anti-inflammatory properties and usefulness in various gastrointestinal disorders (Mehmood and Gilani, 2010). Piperine is recognized as one of the most potent bioavailability enhancers. Studies have demonstrated that piperine can enhance bioavailability (30–200%) of wide range of drugs and nutrients (Atal and Bedi, 2010). Hepatoprotective activity of piperine at a dose of 25 mg/kg has also been reported (Koul and Kapila, 1993; Sabina et al., 2010).

Hepatotoxicity induced by paracetamol (PCM) overdose is a commonly used experimental model to assess hepatoprotective activity of new pharmacological agents (Hussain et al., 2014; Abirami et al., 2015). PCM is regarded as a safe drug at the therapeutic dose but at higher doses, PCM can cause centrilobular necrosis that eventually leads to liver failure (Lee et al., 1984). The major advantage of PCM model is that it is a clinically relevant model and is a dose dependent hepatotoxicant (Jaeschke et al., 2011). The major portion of PCM dose is conjugated with glucuronic acid or sulfate and the rest is converted into reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) through cytochrome P450 enzymes (Nelson, 1990). At therapeutic dose, NAPQI is conjugated with reduced glutathione to form mercapturic acid which is excreted in urine (Mitchell et al., 1973). However, in case of overdose, excess NAPQI depletes GSH content and binds covalently to hepatic cellular proteins resulting in mitochondrial dysfunction and mitochondrial oxidative stress that eventually induces necrosis and apoptosis of hepatocytes (Bhattacharyya et al., 2013; Abirami et al., 2015).

With this background, the current study was planned to explore hepatoprotective effect of *A. marmelos* leaves extract in PCM model alone and in combination with low dose (non-hepatoprotective) of piperine.

Material and methods

Aegle marmelos *extract preparation*

The leaves of *Aegle marmelos* (L.) Corrêa, Rutaceae, were collected from areas in and around Chandigarh, India during the month of January. The plant material was authenticated by Dr. Sujata Bhattacharya, Assistant Professor, School of Biological and Environmental Sciences, Shoolini University, Solan. Voucher specimens of the plant (SUBMS/89) were deposited in the School of Biological and Environmental Sciences, Shoolini University, Solan. The dried coarsely powdered leaves (500 g) were first extracted with petroleum ether followed by 70% ethanol by hot extraction process. The solvent was removed under reduced pressure after completion of extraction process and the extract was stored in vacuum desiccator till further use.

HPLC analysis of Aegle marmelos *extract*

The *A. marmelos* extract was chromatographically analyzed and the rutin content was determined using RP-HPLC (Zu et al., 2006). The HPLC system of Agilent technologies composed of bin pumps combined with Agilent technologies ALS with photodiode array detector using, with column from Agilent eclipse XBD® C 18 bonded with 5 μm (4.6 mm × 150 mm). The method was validated and system suitability parameter was calculated by taking percent RSD of the five standards injections using the same concentration of rutin by HPLC method. Limits of detection and quantification were calculated by a method based on standard deviation (σ) and slope (S) of calibration plot using the formula LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$. The finest resolution and sensitivity of the method was obtained for rutin at 257 mm.

Experimental protocol

Wistar albino rats weighing 180–200 g were used in the study. The animals were housed in temperature controlled (25 ± 1 °C) environment and provided free access to pellet food and drinking water. Animals were acclimatized to laboratory conditions one week prior to start of experiments. Institute Animal Ethics Committee (1201/a/08/CPCSEA) approved the study protocol. Rats were randomly divided into seven groups of six animals each, (i) normal control group (2 ml/kg distilled water), (ii) PCM group (400 mg/kg PCM), (iii) silymarin group (positive control, PCM 400 mg/kg + silymarin 200 mg/kg, (iv) *A. marmelos* extract-25 group (PCM 400 mg/kg + extract-25 mg/kg), (v) extract-50 group (PCM 400 mg/kg + extract-50 mg/kg), (vi) extract-100 group (PCM 400 mg/kg + extract-100 mg/kg) and (vii) extract-25 + piperine group (PCM 400 mg/kg + extract-25 mg/kg + piperine 20 mg/kg). PCM was administered orally in a dose of 400 mg/kg for seven days for the induction of hepatotoxicity (Ravindran et al., 2013). The high dose of *A. marmelos* (100 mg/kg) used in the present study was based on the LD_{50} values (Veerappan et al., 2007). Further, a low dose of 25 mg/kg and intermediate dose of 50 mg/kg were selected. Silymarin was used at a dose of 200 mg/kg (Mahmood et al., 2014) and piperine at 20 mg/kg (Bano et al., 1991; Hiwale et al., 2002). Piperine is known to exert hepatoprotective effect at a dose of 25 mg/kg. Lower dose is inactive and in the current study, lower dose of both piperine and *A. marmelos* was combined to explore the augmentation of hepatoprotective effect with piperine co-administration. The *A. marmelos* extract and piperine were suspended separately in carboxy methyl cellulose and administered through oral gavage separately. All the drugs were administered 30 min before PCM administration orally once daily for seven days.

On the day of sacrifice 2 h after PCM administration rats were injected with thiopentone (50 mg/kg *i.p.*), and blood was withdrawn by cardiac puncture. The serum was separated by centrifugation at 4000 × *g* for 15 min at 4 °C. The liver was rapidly removed and washed in ice-cold saline solution. A part of liver was homogenized in phosphate buffer saline (0.1 M PBS, pH 7.4). The homogenates were centrifuged at 4000 × *g* for 20 min at 4 °C, and supernatant was stored at −80 °C for biochemical estimation. The second part of liver was used for determination of IL-10 and TNF-α level by ELISA and the third was stored in 10% neutral buffered formalin for histopathological study.

Hepatic damage serum biomarkers

Serum biomarkers, alkaline phosphatase (ALP), bilirubin, lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by an auto-analyzer using the Accurex kits (Accurex Biomedical Pvt. Ltd, India).

Oxidative stress parameters

The liver homogenates were used for evaluation of oxidative stress parameters. Malondialdehyde (MDA) level in the liver was

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