

MODULATORY EFFICACY OF *PHYLLANTHUS EMBLICA* L. FRUIT EXTRACT IN COMBINATION WITH CISPLATIN ON XENOBIOTIC METABOLIZING ENZYMES STATUS AGAINST ORAL CARCINOGENESIS MODEL

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ABSTRACT

Objective: The aim of our current study is to evaluate the modulatory efficacy of combined therapy on xenobiotic metabolizing enzymes against oral cancer model.

Methods: Buccal pouch carcinoma was induced in hamsters by painting with DMBA (0.5% in mineral oil) on the left buccal pouch three times a week for 10 weeks.

Results: The results showed that depleted activities of phase II enzymes and significant elevation was observed in the levels of phase I enzymes (cytochrome P450 and b5) in DMBA group of buccal pouch. After supplementation with combination therapy significantly brought back the status to near normal.

Conclusion: The data obtained in this study clearly indicate that amla fruit extract shows therapeutic effect against DMBA-induced oral carcinogenesis.

Keywords: *Phyllanthus emblica*, Cisplatin, Oral carcinogenesis, 7,12-dimethylbenz[a]anthracene (DMBA).

INTRODUCTION

Oral cancer is a frequent neoplasm worldwide, particularly in developing countries. In the United States, oral cancer incidence and mortality rates have been increasing in recent years. Approximately 75% of all oral cancers arise in association with alcohol and tobacco use [1]. The development of oral cancer is a multistep process requiring initiation, promotion and progression. The polycyclic aromatic hydrocarbon, dimethylbenz[a]anthracene is an indirect acting carcinogen that requires metabolic activation to yield an ultimate carcinogenic form [2]. The DMBA-induced hamster buccal pouch model has been well recognized as an excellent animal model for investigations into the development and progression of a number of human malignancies. Despite anatomical and histological differences between pouch mucosa and human buccal tissue, experimental carcinogenesis in this model induced premalignant changes and carcinomas that resemble those that occur during analogous carcinogenic progression in human buccal tissue [3].

Screening of crude extracts of several edible plants revealed the mechanism of several compounds acting together in additive, synergistic or antagonistic manner to suppress tumor promotion [4]. *Phyllanthus emblica* L. (Euphorbiaceae) commonly known as amla, is one of the most versatile medicinal plants that have attracted the focus of attention owing to its medicinal properties. Over the past decades, amla is one of the most popular fruit and is characterized by a high content of vitamin C and other phytochemicals. Amla has been used for the treatment of several disorders such as common cold, scurvy, cancer and heart diseases [5]. Our recent review paper on *Pemblica* has been described to possess anticancer, antihyperlipidemic and hepatoprotective activities [6]. Preceding investigations in our laboratory have demonstrated that chemopreventive potential of garlic oil on hamster buccal pouch (HBP) carcinogenesis model [7]. No detailed scientific studies investigating the effect of amla on oral cancer have yet been conducted. Within this context, the major goal of our research work was performed to envisage the effects of PFMet on *in vitro* antioxidants assay, and xenobiotic metabolizing enzymes against DMBA-induced buccal pouch carcinogenesis.

MATERIALS AND METHODS

Plant material and preparation of amla methanolic fruit extract

Fresh fruits of *Phyllanthus emblica* (amla) were collected from in and around areas of Chidambaram, Tamil Nadu, India. The plant was

taxonomically identified and authenticated by Dr.V.Venkatesalu, Professor in the Department of Botany, Annamalai University. A voucher specimen (No. 207) has been preserved at the herbarium of Botany, Annamalai University. *P.emblica* fruits were washed with water and dried in shade condition. The methanolic fruit extracts of amla was prepared according to the procedure as described in previous communication.

Chemicals and reagents

7,12-Dimethylbenz(a)anthracene (DMBA), mineral oil were obtained from Sigma-Aldrich Chemical Co (USA). All reagents used in the study were of analytical grade.

In vitro antioxidant assays

ABTS radical scavenging assay

The radical scavenging activity of the PFMet was determined by Wolfenden & Willson method [8]. 7mM ABTS radical cation solution was prepared by mixing with 2.5mM potassium persulfate. The reaction mixture in a total volume of 3ml contained extract solution at different concentrations (2-10 µg/ml) was mixed with ABTS solution (2.7 ml), the reaction mixture was allowed to stand for 30 min, and absorbance was recorded at 734 nm. BHT was used as reference. The radical scavenging was calculated using the equation.

$$\text{Scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}} \times 100}{A_{\text{control}}}$$

Where A_{control} = absorbance of the control and A_{sample} = absorbance of the extract or standard.

DPPH free radical scavenging activity

The free radical scavenging activity of the PFMet was measured according to the method of Shimada et al. (1992) [9]. Briefly, 1ml of the extract solution in methanol at various concentrations (10-50µg/ml) was added to 1ml of 0.5 mM solution of DPPH in ethanol and 2 ml of 0.1M acetate buffer (pH = 5.5). The solutions were kept at room temperature for 30 min and then the absorbance was measured at 517 nm by using a UV-Visible Spectrophotometer. Decreased absorbance of the reaction mixture indicated higher free radical scavenging activity. BHT was used as positive control. The DPPH scavenging activity was calculated using the equation as mentioned for ABTS.

Reducing power

The reducing power of PFMet was determined by the method of Oyaizu [10]. Different concentrations of extracts (50-250 μ g/ml) in 1ml of methanol were mixed with 2.5 ml of 0.2 M/L phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After incubation, 2.5 mL of 10% TCA was added and centrifuged at 3000rpm for 10 min. After centrifugation, 0.5 mL of supernatant was mixed with 1 mL of distilled water and 0.5 mL of 0.1% ferric chloride and the absorbance was measured at 700 nm in a spectrophotometer. Vitamin E was used as standard. Increased absorbance of the reaction mixture associates with greater reducing power.

In vivo studies

Animals

Six-10 weeks old male Syrian hamsters (weighed 80-150g) were purchased from National Institute of Nutrition (NIN), Hyderabad. All animals were housed in six per polypropylene cage under controlled environmental temperature with a 12 h light/dark cycle (22 \pm 2°C) and relative humidity (55 \pm 10%) and provided with free access to pellet food and water ad libitum. The standard pellet diet comprised of crude oil (4.12%), crude fibre (4.12%), crude protein (22.12%), ash (5.17%), and sand silica (1.13%) with energy value of 3625 Kcal/kg. Animal care and treatment was performed in accordance with the guidelines of the Indian National law on animal care and use and approved by the Institutional Animal ethical committee at faculty of Medicine, Annamalai University (Reg.no.160/1999/CPCSEA, proposal no.732).

Treatment regimen

Our previous report documented that the chemopreventive efficacy of *Phyllanthus emblica* fruit methanolic extract was investigated using a well established DMBA-induced buccal pouch carcinogenesis model in hamsters [11]. In this study, the animals were allocated into 6 groups of 6 animals each. The total experiment was conducted over an era of 18 weeks. Group 1 served as control group, which were treated with mineral oil only. Group 2 received *Phyllanthus emblica* fruit methanolic extract (PFMet) alone thrice a week for a period of 18 weeks. The left buccal pouches of animals in group 3 were painted with 0.5% DMBA in mineral oil using a no.4 brush thrice a week for 10 weeks. Each application leaves 0.4ml of solution. Tumor bearing hamsters in group 4 were given an oral administration of PFMet (200mg/kg) extract alone thrice a week for 8 weeks starting from the 10th week of tumor formation. The hamsters in group 5 received an intraperitoneal injection of Cis (2mg/kg body weight) once a week for 8 weeks starting from the

10th week of tumor formation. Animals in group 6 were treated with combinations of amla (200mg/kg BW) and Cis (2mg/kg BW) as described in group 4 and group 5. The experiment was terminated at the end of 18 weeks and animals were sacrificed by cervical decapitation.

Biochemical estimations

The activities of glutathione-S-transferase (GST) and DT-diaphorase in liver was measured according to the method of Habig et al. [12] and Ernster [13]. Cytochrome P450 and b5 levels in the liver were assayed by the method of Omura et al. [14]. Glutathione reductase (GR) activity in liver was estimated by the method of Carlberg et al. [15]. The level of GSH in liver was assayed by the methodology of [16].

Statistical Analysis

Results were expressed as mean \pm S.D. The significant difference between the groups was statistically assessed by Duncan's Multiple Range Test (DMRT) using SPSS 11.0 software package. $P < 0.05$ was considered statistically significant.

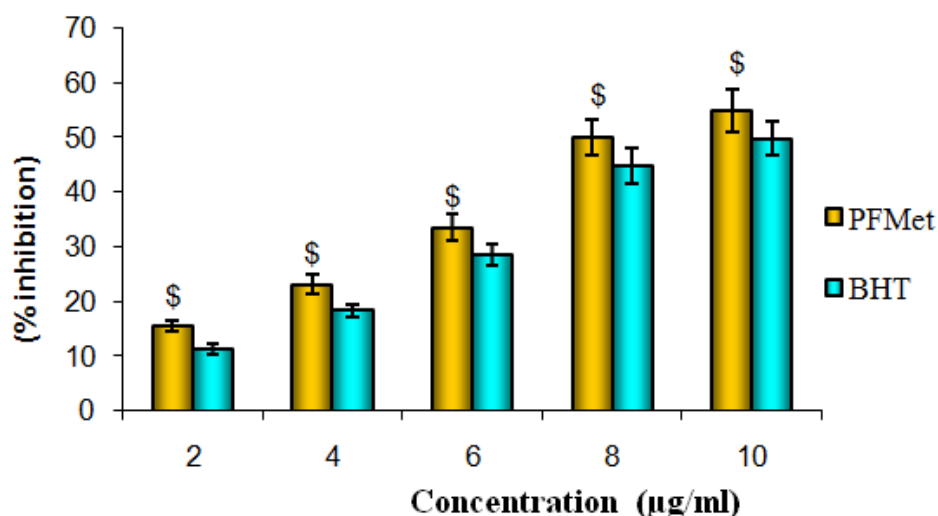
RESULTS

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen donating antioxidants and of chain breaking antioxidants and to measure the relative antioxidant ability to scavenge the ABTS⁺ radical. The effect of different concentrations (2-10 μ g/ml) of extract on the inhibition of ABTS radical in comparison with BHT was shown in (fig. 1A). From our investigations on the ABTS radical scavenging activity, it was found that the PFMet inhibited ABTS radicals in a dose dependent manner. At 10 μ g/ml concentration, PFMet showed a higher free radical scavenging activity (54%) than the standard antioxidant BHT.

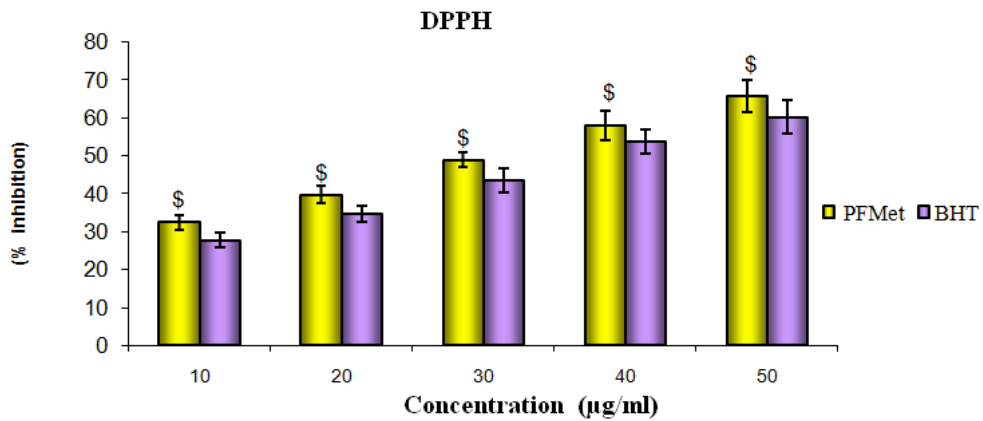
The DPPH radical is commonly used for the assessment of antioxidant potency in vitro and is foreign to biological systems. A DPPH radical represents the powerful stable free radicals with a characteristic absorption maximum between 515 and 517 nm, which is decreased in the presence of antioxidants capable of reducing it to its hydrazine form through hydrogen or electron transfer reaction. The maximum DPPH radical scavenging activity was observed at a concentration of 50 μ g/ml in comparison with the standard BHT (Fig 1B).

Reducing power assay is frequently used to assess the electron donating capacity of natural antioxidants. As can be seen from (fig. 1C), the extract had the reducing power in a dose dependent manner (100-250 μ g/ml). Increased concentration of the extract indicates reducing power. Reducing power of PFMet was found to be closer when compared with synthetic vitamin E.

(A) ABTS



(B) DPPH



(C) Reducing Power

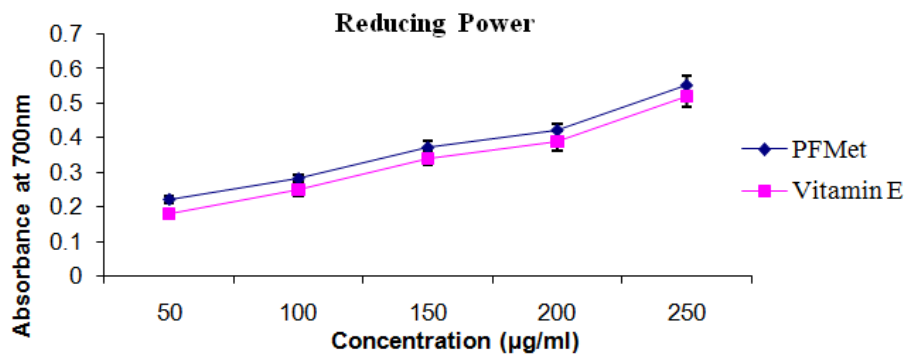


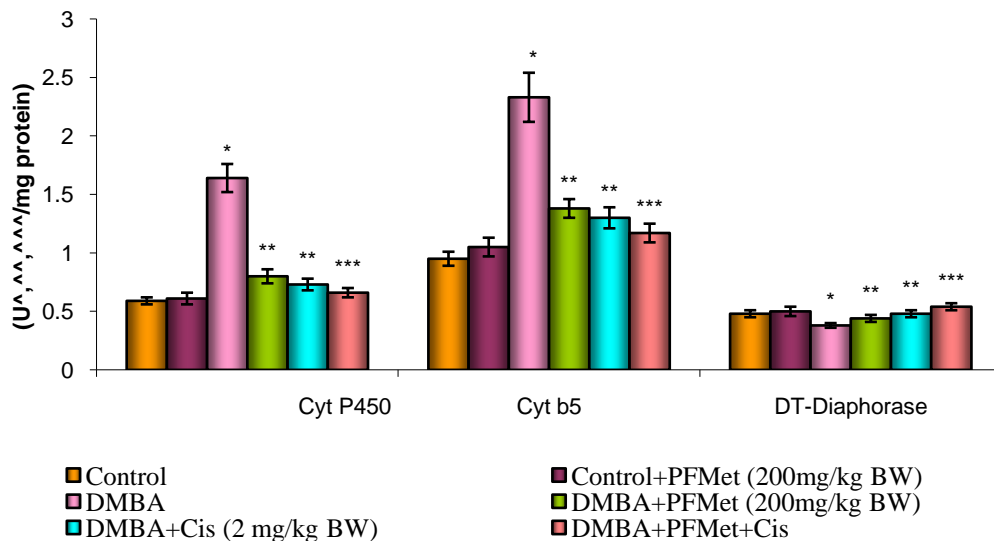
Fig. 1: Scavenging activities of various concentrations of *Phyllanthus emblica* fruit extract on ABTS⁺ radical (A) and DPPH radical (B). Values were given as represented as mean ± SD of six experiments in each group. *P<0.05 - significantly different from BHT.

IN VIVO

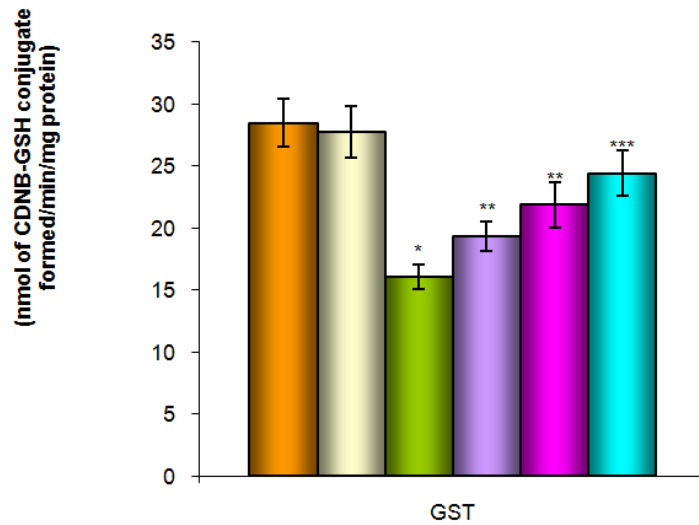
Fig 2A-D summarizes the activities of the phase I (cytochrome P₄₅₀ and b5) and phase II (GST, GR, GSH, DT-Diaphorase) enzymes in the liver of control and experimental groups. While the DMBA treated group revealed higher levels of phase I enzymes and lower levels of phase II enzymes when compared with control group.

Supplementation with amla, cis and combination significantly reduced the phase I enzymes and augment the phase II enzymes level. The effect being greater in the combination group than either PFMet or cisplatin alone. Although, control and PFMet alone treated group showed no significant changes in the levels of liver phase I and phase II enzymes. (P<0.05).

(A)

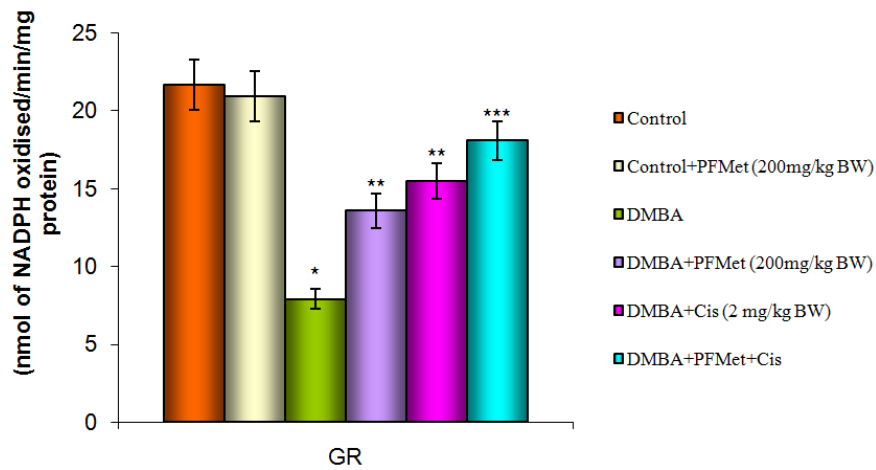


(B)



Each bar represents mean \pm S.D. from 6 rats. Values not sharing a common superscript at $P < 0.05$ (DMRT). U[^] - micromoles of cytochrome P₄₅₀; U^{^^} - micromoles of cytochrome b5; U^{^^^} - micromoles of 2,6-dichlorophenol reduced per minute. * $P < 0.05$ - significantly different from control group, ** $P < 0.05$ - significantly different from DMBA-treated group, *** $P < 0.05$ significantly different from amla or Cis individual treatment groups.

(C)



(D)

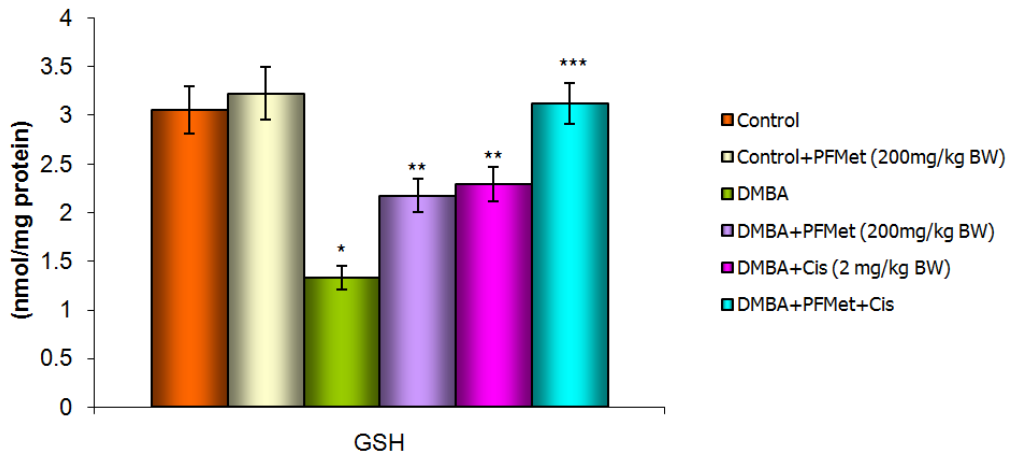


Fig. 2: Combined effects of PFMet and Cis on the levels of phase I (cytochrome P450 and cytochrome b5) and phase II enzymes DT-Diaphorase, GST, GR and GSH levels in liver of control and experimental animals.

Each bar represents mean \pm S.D. from 6 rats. Values not sharing a common superscript at $P < 0.05$ (DMRT). * $P < 0.05$ - significantly different from control group, ** $P < 0.05$ - significantly different from DMBA-treated group, *** $P < 0.05$ - significantly different from amla or Cis individual treatment groups.

DISCUSSION

The cytochrome P450 enzymes (cytochrome P450 and cytochrome b5) refers to a superfamily enzyme that activates oxidative damage, thus modulating their toxicity as well as involved in the excretion of most carcinogens [17]. GST is a multifunctional proteins encoded by multigene family that may protect the cell from oxidative damage via elimination of electrophilic compounds. GST is involved in the detoxification of the epoxides and other reactive intermediate metabolites produced during carcinogenic process induced by DMBA [18]. An antioxidant enzyme, glutathione reductase catalyze the conversion of glutathione disulphide to glutathione via reduction of NADPH-dependent enzyme, and consequently maintaining the levels of GSH in the cell [19]. DT-diaphorase is an inducible enzyme that may protect cells against the toxicity of xenobiotics, and catalyses the two-electron reduction of quinones and other nitrogen oxides [20, 21]. DTD also prevents the formation of semiquinones by one electron redox cycling and subsequently reduces the generation of free radical through the dismutation of semiquinone [22]. In our investigation, we found that the cytochrome P₄₅₀ and cytochrome b5 were significantly increased and GST, GR, GSH and DT-Diaphorase activities were significantly decreased in liver of tumor hamsters, whereas after treatment with PFMet, Cisplatin, combination significantly restored the levels of above mentioned phase I and phase II enzymes to near normal, which is line with previous investigation [23]. It should be emphasized that the most significant phase II enzymes such as GST and DTD are involved in the detoxification of carcinogenesis. Thus, amla acts as a chemotherapeutic agent by reducing the levels of free radicals induced damage and contributes to inhibition of oral carcinogenesis.

GSH is the foremost intracellular thiol and ROS scavenger [24]. Reduced glutathione enzymes is an important cofactor for GSH transferases, GSH peroxidase, GSSG reductase system, protects cells against oxidative stress via glutathione redox cycle and also detoxifies reactive oxygen species generated free radicals from exposure to xenobiotics including carcinogens [25]. In the present investigation, the levels of GSH were decreased in liver and increased in buccal pouch animals exposed to DMBA, while amla, cis and combination administration modulate the GSH levels to near normal. In cancerous condition, the thiol group plays a pivotal role in the protection against harmful effects of free radicals. Significant decreases in DMBA liver of GSH level which may be due to increased utilization of GSH for scavenging the free radicals. Our observation is in line with previous findings [26]. The significant inhibition of phase I enzymes and induction of phase II enzymes by amla and cisplatin may protect against carcinogenesis metabolized by these enzymes.

Amla fruit has been considered a healthy functional food contains abundant levels of vitamin C and bioactive phenolic phytochemicals such as gallic acid, ellagic acid, quercetin, kaempferol, which possess diverse health benefits. HPLC analysis of amla fruit extract revealed the presence of ascorbic acid, gallic acid, ellagic acid and quercetin. The synergistic effect observed in the present study has been attributed to abundance of diverse phenolic phytochemicals constituents present in the amla fruit extract. Our results confirm the hypothesis proposed by De Kok et al. (2008) [27] shown that various phytochemicals interact with another to produce synergistic antiproliferative and cytotoxic effects. Phenolic acids and flavonoids compounds present in natural food products have focussed great attention recently owing to its potent antioxidant property. The highest antioxidant activity of amla fruit extracts which may be due to the presence of compounds like gallic acid or epigallocatechin in substitution with 3-vicinal hydroxyl groups on the B ring of phenolic acids or flavonoid compounds [28]. Our observation supports the hypothesis that the protective effect of amla fruit extract against the anticancer drugs induced toxicities in hamsters.

CONCLUSION

The identification of natural products has been emerged as the best candidate for oral cancer therapy and can improve cancer chemotherapeutic effect and diminishing the non-specific toxic effects of anticancer drugs. The present study concludes that amla fruit extract has chemotherapeutic effect when administered along

with cisplatin via modulation of detoxification cascades during DMBA-induced buccal pouch carcinogenesis. Hence, amla fruit extract might have a promising role in reducing the toxicity of chemotherapeutic drug cisplatin and provide useful data for the improvement of nutritional strategies in reducing oral cancer threat.

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