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Protective Effect of Apigenin from *Morus indica*.L against Methylglyoxal Induced Oxidative DNA Damage

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Abstract
Methylglyoxal is reported to induce oxidative stress and glycation. Apigenin from *Morus indica* has potent antiglycation effect. Hence, the objective was to examine the effect of Apigenin derived from *Morus Indica* on oxidative DNA damage caused by the reaction of methylglyoxal with lysine. DNA strand breakage was investigated by Gel electrophoresis to determine the effect of apigenin on calf thymus DNA incubated with methylglyoxal and lysine. In addition, the effect of methylglyoxal on morphology of Red blood corpuscles using apigenin was also characterized by scanning electron microscope by *ex-vivo* assay. Apigenin prevented methylglyoxal induced oxidative stress and DNA damage in the presence of copper and also protected rupture of red blood corpuscles. The oxidative stress plays a role in methylglyoxal induced cell injury and that apigenin blocks this effect by virtue of its antioxidant property.

Keywords
Apigenin, Mulberry, Oxidative stress, DNA damage, RBCs.

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INTRODUCTION
Methylglyoxal (MGO), a highly reactive di-carbonyl compound, is the major precursor in the formation of advanced glycation end products (AGEs) [1]. MGO is mainly formed as a by-product of glycolysis and is also formed in various food stuffs during processing and fermentation [2]. Different amino acids particularly lysine and arginine reacts with MGO to form non enzymatic protein glycation and form AGEs products. Accumulation of these products in excess may increase the complications of diabetes and other age-related diseases, as the resulting reaction AGEs alters the physicochemical and biochemical properties of proteins [3]. Recent *in-vitro* studies have focused interest on the formation of reactive oxygen species (ROS) and oxidative stress with MGO which results in increase protein carbonyl formation and thus reduces thiol-containing protein [4]. Together ROS and oxidative stress can contribute to damage many biological systems including DNA and cause cell apoptosis, cell injuries [5]. Under physiological circumstances, MGO is detoxified by the glyoxalase system into D-lactate, with glyoxalase I as the key enzyme in the anti-glycation defence. In this regard, inhibitor of AGEs known for the prevention of AGEs related diseases has emerged as a new strategy. Both natural and synthetic compounds have been evaluated for inhibiting the AGEs reactions in biological systems. Thus, identification of AGEs inhibitors particularly from

natural products is the current area of research. Previous studies on MI have been evaluated for its antioxidant [6], antihyperglycemic [7] acute toxicity [8] and antihyperlipidemic potential [9]. The effect of 80 % aqueous methanol extract of leaves of three commercial varieties of MI (G4, V1 and S36) and two wild varieties of Morus sp., (*Morus laevigata* and *Morus serrata*) on *in vitro* protein glycation in BSA-glucose model were screened [10]. Among them, MI-G4 variety exhibited the highest AGEs inhibition and metal ion chelation. The results have given substantial evidence for the presence of polyphenols from MI-G4 variety. Thus, apigenin from MI-G4 variety was further extracted and quantified by UP-LCMS, isolated by preparative HPLC, characterized by FTIR, NMR and SEM and has shown potential anti-glycation effect in all the different stages of protein glycation (unpublished data). In addition, API is also proved to inhibit Aldose reductase (ALR) activity, one of the major complications of diabetes (cataract) in lens [11]. Based on the above findings, the aim of the current work was to investigate the protective effect of API extracted from MI-G4 on oxidative DNA damage caused by the reaction of MGO with lysine in presence of copper (Cu^{2+}). In addition, the effect of MGO on morphology on RBCs using API was also characterized by SEM by *ex-vivo* assay.

MATERIALS AND METHODS

Experimental site: The experiment was carried out in the Department of Studies in Food Science and Nutrition, University of Mysore, Manasagangotri, Mysuru, Karnataka, India between March 2017 and May-2018.

Plant materials

The leaves of commercial varieties of *Morus indica* G4 (ISGR Reg. No.: 050564), were collected in the month of May-2016 from Central Sericulture Research and Training Institute (CSRTI), Mysore. Apigenin was isolated from methanol extract of leaves by preparative HPLC and characterized through UP-LCMS, NMR, FTIR and SEM (unpublished data).

Chemicals and reagents

Methylglyoxal (40% in water), Aminoguanidine hydrochloride, Calf thymus DNA, L-lysine, Copper were purchased from Himedia (India). All other chemicals used were of analytical grade.

Effect of API on MGO/Lysine induced DNA strand breakage

The assessment of DNA strand breakage was performed according to Satish *et al* with slight modification [12]. Calf thymus DNA (0.5 µg) was incubated with 5 mmol/L lysine and 5 mmol/L MGO in the presence of Cu^{2+} for 3hrs at 37°C. To stop the reactions, samples were frozen at -70°C. Following the reaction, was resolved by 1% agarose gel electrophoresis, visualized by ethidium bromide staining in Bio-rad XR+ gel documentation system.

Protective effect on RBC structural morphology

Blood sample (2 ml) was collected from a healthy individual in citrate-containing tubes. The sample preparation was done as described by Buys *et al* [13]. In brief, the blood samples were centrifuged (1500x g,5 min) at 4 °C, RBCs were separated from the plasma and buffy coat, and the pellet containing RBCs were washed thrice with 20 mM phosphate buffered saline (pH7.4). These RBCs (50 µl) were treated with MGO (5 µM) both in the presence and absence of 15mM API and allowed to react for 1 h at 37 °C. Then, the reaction mixture was centrifuged (RBCs) and washed thrice with PBS. Then, the cells were fixed with 2.5% glutaraldehyde for 30 min followed by centrifugation (1000 rpm, 30 min) to remove the glutaraldehyde and washed thrice with PBS. 5 µl suspension of RBCs were placed on an aluminium foil and allowed to dry overnight. The morphological feature of the RBCs was examined under scanning microscope (ZEISS, Germany) at 15 kV.

RESULTS

Effect of API on MGO/Lysine induced DNA strand breakage

To understand the oxidative damage of DNA by the reaction of methylglyoxal with lysine we performed Gel electrophoresis for CT-DNA as shown in (Fig. 1). The undamaged CT-DNA cleavage was observed in lane 1 (only CT-DNA), lane 2 (CT-DNA + lysine), lane 3 (CT-DNA + lysine +MGO). Whereas, in lane 4 (CT-DNA incubated with lysine + MGO + Cu^2) markedly induced DNA stand breakage. Interestingly, in the presence of AG, in lane 5 (CT-DNA incubated with lysine + MGO + Cu^{2+}) and API in lane 6 (CT-DNA incubated with lysine + MGO + Cu^{2+}) were DNA strand breakage was inhibited.

From DNA Gel images, the relative front (R_f) and percentages (%) of band intensity were expressed by using Bio-rad XR^+ gel documentation system as shown in (Fig. 2). The undamaged CT-DNA cleavage was observed in lane 1 (only CT-DNA) with R_f value (0.75) and band intensity (98.6%), lane 2 (CT-DNA + lysine) with R_f value (0.74) and band intensity (97.3%), lane 3 (CT-DNA + lysine +MGO) with R_f value (0.74) and band intensity (95.6%), whereas, in lane 4 CT-DNA incubated with lysine + MGO + Cu^{2+}, a decrease in R_f value (0.60) and band intensity (90.9%) was observed, when compared with undamaged

DNA cleavage (lane 1-3). In the presence of AG and API, in lane 5 (CT-DNA incubated with lysine + MGO + Cu^{2+}) and lane 6 (CT-DNA incubated with lysine + MGO + Cu^{2+}), the R_f values (0.72), (0.70) and band intensity (95.3%), (95.1%) respectively, was comparable with the values observed in lane 1- 3 of undamaged DNA (Fig.2)

Protective effect on RBC structural morphology

Scanning electron micrographs of RBCs treated *in vitro* with MGO and API are shown in Fig. 3. Normal red blood corpuscles showed typical biconcave shape (Fig. 3A). RBCs treated with MGO appeared as discocytes resulted in the formation of echinocytes (Fig. 3B). The morphological changes induced by oxidative system were prevented when the cells were treated with AG (Fig. 3C) and API (Fig. 3D).

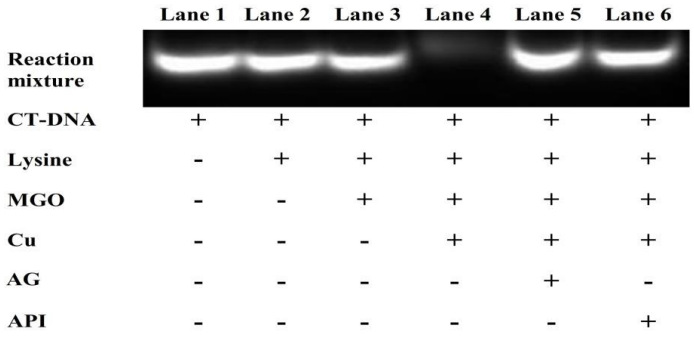

Fig.1: Effect of apigenin on methylglyoxal/lysine-induced DNA strand breakage in the absence or presence of copper. Lane 1: CT-DNA, Lane 2: CT-DNA+Lysine, Lane 3: CT-DNA+Lysine+Methylglyoxal, Lane 4: CT-DNA+Lysine+Methylglyoxal+Copper, Lane 5: CT-DNA+Lysine+Methylglyoxal+Copper+ aminoguanidine, Lane 6: CT-DNA+Lysine+Methylglyoxal+Copper+Apigenin

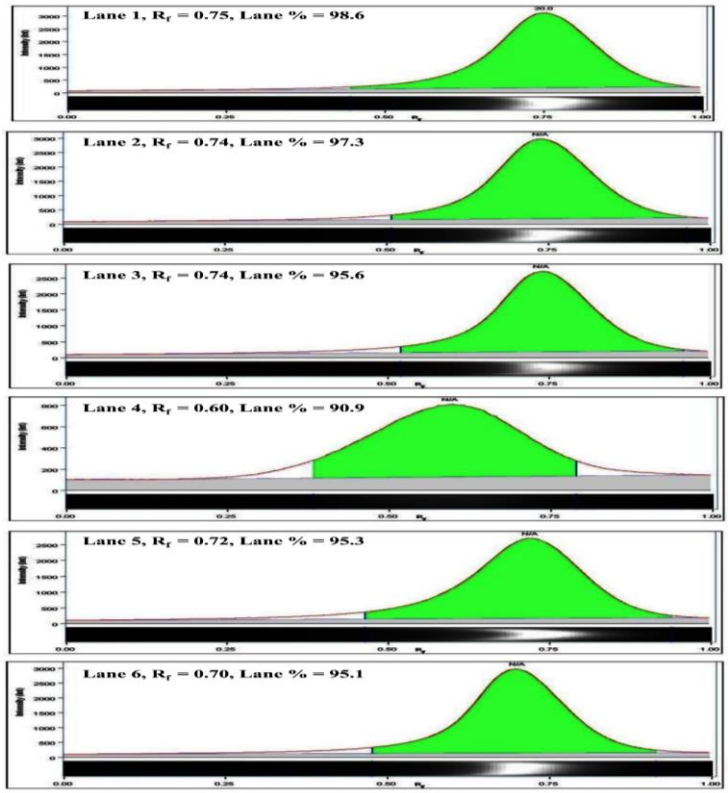

Fig.2: Gel images of the relative front (R_f) and percentages of band intensity were expressed by using Bio-rad XR$^+$ gel documentation system. Lane 1: CT-DNA, Lane 2: CT-DNA+Lysine, Lane 3: CT-DNA+Lysine+Methylglyoxal, Lane 4: CT-DNA+Lysine+Methylglyoxal+Copper, Lane 5: CT-DNA+Lysine+Methylglyoxal+Copper+aminoguanidine, Lane 6: CT-DNA+Lysine+Methylglyoxal+Copper+Apigenin.

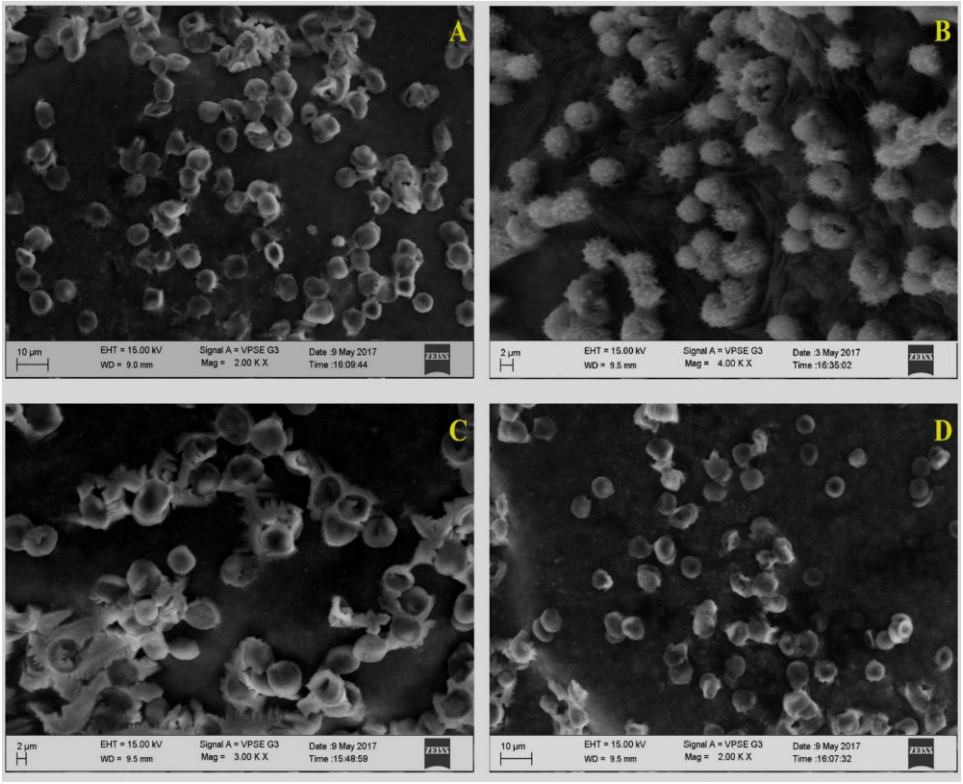

Fig.3: Scanning electron micrograph. (A) Normal Red blood corpuscles showing typical biconcave shaped. (B) Methylglyoxal on Red blood corpuscles showing the loss of biconcave shape. (C) and (D) Protective effect amino guanidine and apigenin against methylglyoxal induced oxidative damage on red blood corpuscles respectively.

DISCUSSION

Several diverse biological processes including age related diseases, mutagenesis and carcinogenesis have been developed due to oxidative DNA damage from ROS [14]. Among these, diabetes is the degenerative disorder characterised by the macro vascular and micro vascular complications [15]. One of the major causative factors is formation of AGEs. Therefore, there is a need to detect novel approaches to prevent these AGEs. As synthetic products may end up in formation of side effects, focus research area should be on the naturally occurring compounds that have the potential to prevent these reactions [16]. In this regard, the present study is first of its kind to determine the effect of API from MI on oxidative damage of DNA by the reaction of MGO with lysine. When MGO reacts with lysine in the presence Cu^{2+} the reaction leads to the production of free radicals [17]. Studies report that Cu^{2+} has been shown to enhance DNA damage caused by the glycation reaction *in vitro* [18]. These results suggest that superoxide anion and H_2O_2 may generate from the glycation reaction of MGO with lysine and then Cu^{2+} likely participates in a Fenton's type reaction to produce hydroxyl radicals, which may cause DNA cleavage [19]. The present results showed that the incubation of DNA with API/MGO/lysine/ Cu^{2+} for 3 hrs did not induce strand breakage. The inhibition of DNA strand breakage exhibited by API is attributed to its rich in polyphenol and antioxidant capacity as reported in the earlier studies [6]. The results are in accordance with other naturally occurring compounds viz; curcumin, isoferulic acid. Meeprom et al., reported that isoferulic acid, a derivative of cinnamic acid prevented MGO/lysine-mediated oxidative DNA damage in the presence and absence of copper ion. The protective ability of isoferulic acid was directly correlated to inhibition of hydroxyl and superoxide anion radical generation during the reaction of MGO and lysine [20]. Chang et al. revealed that curcumin also prevented MGO/lysine-induced oxidative stress and DNA damage [21]. Studies indicate that naturally occurring compounds are preferable than the synthetic products because of their limitations (high cost, GI disturbances, liver toxicity) in preventing AGEs and its complications [22]. In addition to them preferability, it is also important to screen them for their dosage, bioavailability and toxicity levels before recommending for therapeutic use [23]. Studies have reported acute toxicity levels of MI in rats and found to be non-toxic. Based on these finding, API is a

natural compound and it has already been screened for toxicity effects, however, pharmacological investigations using advanced techniques are essential to validate the potential of apigenin derived from *Morus indica* in the prevention of oxidative stress.

MGO is membrane permeable and can readily cross any cell membranes without any cofactors or transporters [24]. The increase in concentration of MGO above the permissible level in pathological conditions due to the formation of its precursor molecules leads to the disruption and the change in shape of RBCs eventually leading to the loss of its function of oxygen transport protein haemoglobin which ultimately results in oxidative stress [13]. The results in the present study indicate that API prevents the morphological changes induced by oxidants in RBCs. It is clearly evident from studies that hyperglycemic conditions and the accompanying presence of di-carbonyl intermediates such as MGO tend to disturb the normal shape of RBCs [25]. Studies have reported the hypoglycaemic effect of MI in STZ induced diabetic rats [7] and safety of MI as per OECD guidelines. *Morus indica* being a rich source of phytochemicals and economical medicinal plant, can be exploited commercially as nutraceutical / natural supplement/adjuvant in the prevention and management of degenerative diseases.

CONCLUSION

Advanced glycation end-products (AGEs) resulting from non-enzymatic glycation are one of the major factors implicated in diabetic complications. Earlier studies reported the *Morus indica* extract to be non-toxic and prevent glycation. Current research is focused on discovering new-novel compounds that may be used as potential AGEs inhibitors without affecting the normal structure and function of the biomolecules. In the present study, presence of apigenin a bioactive compound in *Morus indica* demonstrated the protection of DNA against MGO induced damage. This is the first study carried out in MI-G4 variety which has proven its potential action by virtue of its antioxidant property, by blocking the formation of ROS, oxidative stress and thus protect DNA damage. As apigenin is a natural compound, its role is almost comparable to the synthetic aminoguanidine. The study highlights the DNA protection of API from MI-G4 variety, thus providing scope for further exploration using *in vivo* models.

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