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Fortification with *Morus indica* extract attenuates the formation of AGEs in bread

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Abstract

Intake of advanced glycation endproducts (AGEs) may be increased by consuming high-sugar meals and foods cooked at high temperatures. AGEs-induced inflammation has been linked to diabetes and degenerative diseases. Leaves from mulberry (*Morus indica* L) (MI) trees have been used in Chinese medicine for over 3000 years and are a functional food because of their phytochemical content. MI extract was used to substitute 0, 0.25, 0.50, 0.75 and 1% of wheat flour for making MI wheat bread (MI-B). 5% of turmeric powdered bread (TM-B) was also prepared and used as a positive control. The bread's overall acceptability was determined using a sensory evaluation on the hedonic test. Further, the antioxidant capacity was assayed by using DPPH method and antiglycation effects of MI extract against the formation of fluorescent AGEs in fortified bread samples. The overall acceptability of bread with MI extract at substitution levels of 0.5% had a good liking score. The total polyphenol content in the MI-B and TM-B was 2.01 mg GAE g^{-1} and 1.54 mg GAE g^{-1} on a dry weight basis. MI-B showed significantly higher antioxidant activities, followed by TM-B > control bread. When compared to TM-B, AGEs inhibited fluorescent formation by 31%. Overall, the findings support MI extract as a functional food ingredient in the bread system, providing consumers with a higher antioxidant intake by depleting AGEs load. However, the stability and reactivity of polyphenols during thermal processing should be considered before commercialization.

Key words: Antiglycation, functional foods, medicinal plant and polyphenol.

Introduction

Thermal processing is an important process in the food industry. The Maillard reaction, which is one of the most common reactions that occur during thermal treatment, not only contributes to the formation of browning colour, aroma and taste compounds, but it also leads to the formation of some unpleasant toxic substances such as acrylamide, heterocyclic amines, and advanced glycation endproducts (AGEs) (Teng *et al.*, 2018). Currently, modern meals are predominantly thermally processed, they are more likely to contain significant levels of toxic aldehydes because of the baking at higher temperatures (Uribarri *et al.*, 2010).

Advanced glycation end products (AGEs) are heterogeneous molecules that arise when a protein or lipid in the bloodstream are exposed to sugar under hyperglycemic conditions. The factors responsible for AGEs formation include ageing, degenerative diseases such as diabetes, atherosclerosis, chronic kidney disease, Alzheimer's disease, *etc.* (Singh *et al.*, 2014). AGEs are formed by a series of events (Maillard Reaction) in which sugars react with free amino groups of peptides, proteins, and amino acids, specifically lysine and arginine residues, to form a ketoamine known as the Amadori product, which is associated with the diabetic complications including retinopathy, cataracts and other health disorders (Sharma *et al.,* 2015).

As AGEs inhibitors, both synthetic compounds and natural extracts/products have been investigated, it has been observed that the inhibitory effects of most natural extracts against the formation of AGEs are primarily due to their high phenolic compound contents (Mildner-Szkudlarz *et al.,* 2017).

Mulberry (*Morus* spp.) is a popular crop in China, India, Thailand, Japan and other Asian countries. Mulberry leaves have been used in traditional Chinese medicine for over 3000 years and are commonly used as silkworm feed (Yu *et al.,* 2018). Mulberry leaves have been identified as an excellent food source because of their high protein, carbohydrate, vitamin, microelement, and dietary fibre content (Srivastava *et al.,* 2016). According to some reports, mulberry leaves contain a high concentration of bioactive compounds such as phenolic acids, flavonoids, alkaloids, and -aminobutyric acid (GABA) (Devi *et al.*, 2013) and their bioactivity was considered highly concerning factors when they were used as functional foods (Yu *et al.,* 2018). Both nutrients and bioactive substances have the potential to improve human health synergistically (Srivastava *et al.,* 2016). Mulberry leaves (*Morus indica.* L) have been screened for various biological properties such as antioxidants, toxicological studies, anti-hypercholesterolemic, and anti-diabetic effects in *in-vitro, ex-vivo* and *in-vivo* models (Arabshahi-Delouee and Urooj, 2017; Reddy *et al.,* 2016; Reddy Palvai and Urooj, 2014; Urooj and Ahmed, 2013).

Bread is a popular staple food that is consumed all across the world (Collar, 2015). Consumers are attracted to the delicious bread crust formed during baking by the Maillard reaction between proteins and sugars. Nevertheless, it is also a rich source of dietary AGEs (dAGEs), which is a potential risk to human health, notably in diabetes patients (Lin and Zhou, 2018). In addition, the intake of dAGEs could enhance the production of intracellular reactive oxygen species, which leads to cellular level damage (Teng *et al.*, 2018; Sharma *et al.,* 2015). The literature also indicates that

polyphenols have been widely incorporated into the dough to prepare various baked food products such as bread, cakes, muffins and cookies to improve their functional properties and reduce the content of AGEs during the baking process (Zamora *et al.,* 2016). In our previous studies, we found that *Morus indica*-G4 (MI-G4) leaf extract is a good source of phytochemicals such as polyphenol and flavonoids and Apigenin (API) was the major phyto-constituent. API had potential antiglycation properties by the different stages of protein glycation (Satish and Urooj, 2019). However, whether MI extract could serve as an antiglycative agent to inhibit the formation of AGEs in bread remains unknown to the best of our knowledge. With this background, the work was aimed to evaluate the antiglycative effects of different addition levels of MI extract in wheat flour against the formation of AGEs in the bread model.

Materials and methods

Ingredients for bread making were purchased from a local supermarket in Kolar, Karnataka.

Chemicals: DPPH (2,2-diphenyl-1- picrylhydrazyl), Gallic acid, Folin–ciocalteau reagent, were purchased from Sigma–Aldrich (India). All other chemicals were analytical grade and purchased from Himedia (India).

Collection of samples: *M. indica* leaves was procured from Central Sericulture Research and Training Institute (CSRTI), Mysore.

Preparation of extract: The leaves were cleaned, cut into small pieces, dried in the oven at 37 °C overnight, powdered and passed through a 60 mesh sieve and stored at 4 °C until further use. Aqueous extract was prepared by extracting the powdered samples (50 g) with distilled water (1:10 w/v) in a mechanical shaker for 24 h at room temperature. The extract was filtered and freeze-dried.

Preparations of bread: For preparation of bread method of Lim *et al*. (2017) was followed. *M. indica* extract substitution levels were 0, 0.25, 0.5, 0.75 and 1% in wheat flour. The raw materials for bread baking were weighed according to the formula proportions. 0, 0.25, 0.5, 0.75 and 1 g of MI extract were incorporated in 100, 99.75, 99.5, 99.25 and 99 g wheat flour, respectively. For positive control, 5g powdered turmeric was incorporated in 95 g of wheat flour, the amounts of other ingredients were similar in these different formulations *i.e.,* 8 g sugar, 2 g salt, 2 g yeast, 3g butter, 2 g non-fat dry milk and 65.0 g water. Bread dough were formed for 6 min at 30 ºC and fermented at 35 ºC for 30 min. Bread was baked at 230-240 ºC in a convection oven for 16 min. Bread was cooled to room temperature for 60 min.

Sensory evaluation: The hedonic test was used to determine the degree of overall acceptability for the bread. The sensory evaluation was carried out with bread sample within 24 h after baking (Larmond, 1997).

Preparation of bread extract for its biological activity: Powdered bread samples (50 g) were extracted with 100 mL of 80 % methanol for 6 h in a mechanical shaker. The extracts were filtered, and filtrates were evaporated at 40 ºC under reduced pressure to dryness in a rotary evaporator (Superfit, India). The residue of each extract was stored in an airtight container at 4 ºC until used to estimate phytochemical content and its biological activity.

Total phenolics content: Total phenolics were determined using the Folin–Ciocalteau method (Waterhouse, 2002). A hundred microliters of this extract was diluted to 3 mL using distilled water and mixed with 0.5 mL Folin–Ciocalteau reagent. After 3 min, 2 mL of 20 % sodium carbonate was added followed by thorough mixing. Solutions were heated in a 40 ºC water bath for 30 min. Absorbance was measured at 765 nm using a spectrophotometer. Results were expressed as mg gallic acid equivalent 100 g^{-1} dry weight.

Antioxidant capacity by DPPH method: The DPPH assay was conducted based on the method (Pyrzynska and Pękal, 2013). The bread extract, Trolox (0.1 mL) and 60 µM methanol DPPH % solution (3.9 mL) were mixed and incubated in the dark for 2 h. The absorbance at 515 nm was measured by the spectrophotometer. The antioxidant capacity was expressed as mg Trolox equivalent per 100 g dried bread sample.

Impact of MI extract on AGEs formation in bread samples: To determine the fluorescent AGEs content of bread samples, 4.75 mL of extraction buffer (0.05 % Tween 20, 1 % SDS, 5 % β-mercaptoethanol and 50 mM Tris-HCl at pH 7.4) was used to extract protein from 250 mg of bread samples. The extraction was conducted gently on a shaker overnight at ambient temperature. After centrifugation for 10 min at 8000 g, the supernatant containing extracted protein (25 mg/mL) was collected and pippeted to a 96-well plate with 100 µL per well. Total AGEs fluorescence was detected with (excitation, 370 nm; emission, 440 nm) wavelength employing a microplate reader as described Zhang *et al.* (2014).

The inhibition of AGEs formation was calculated using the equation,

$$\text{Inhibition of AGEs formation (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Whereas, A_s and A_c represents the relative intensity of AGEs fluorescence of the samples and control, respectively. While the relative intensity of AGEs fluorescence in the control sample was considered as 100%.

Statistical analysis: Statistical analysis was performed using the statistical analysis program (SPSS, 16.0, International Business Machines, USA). Comparisons between groups (control and fortified samples) were performed by one-way ANOVA with Tukey's HSD post hoc test.

Results and discussion

The hedonic test was used to determine the degree of acceptance of bread prepared using different concentrations of MI extract; the results are given in Table 1. The colour of bread with 0.75 and 1 % extract of MI had the lowest liking score. Since the colour of MI extract was light green, the 0.25 % substitution of MI extract did not interfere with the original colour of the bread made with wheat. The taste and overall acceptability of bread with MI extract at substitution levels of 0–0.5 % had a good liking score. The sensory characteristics liking results indicated that a partial replacement of wheat flour with up to 0.5 % MI extract in

bread gives satisfactory overall consumer acceptability. However, the bread which contained 0.75 and 1 % MI extract was rated comparatively lower, which might be due to excessive amounts of phenolic compounds, which can negatively affect the taste of food. This finding is similar to the results of Drewnowski and Gomez-Carneros (2000). Cross-sectional views of control wheat bread (without extract), positive control (turmeric bread prepared with 5 % turmeric powder) and wheat bread prepared with 0.5 % MI extract are presented in Fig. 1.

Table 1. Sensory evaluation of bread prepared by the substitution of wheat flour with *M. indica* extract

Attributes	Substitution level of *Morus indica* extract (%)				
	0	0.25	0.5	0.75	1.00
Colour	9 [a]	8.4 [a]	7.4 [b]	4.4 [c]	2.8 [d]
Aroma	8.8 [a]	8.8 [a]	8.6 [a]	8.0	8.0 [b]
Taste	9.0 [a]	9.0 [a]	8.0 [b]	6.0 [c]	4.0 [d]
Texture	8.8 [a]	8.8 [a]	7.0 [b]	7.0	6.0 [c]
Overall	9.0 [a]	9.0 [a]	8.0 [b]	6.0 [c]	5.0 [d]

Nine-point hedonic scale with 1, 5 and 9 representing extremely dislike, neither like nor a dislike, and extremely like, respectively. The means with different letter superscripts within the same row are significantly different ($P < 0.05$).

Medicinal plants concerning polyphenols are hot research topics worldwide due to their physiological and pharmaceutical activities (Tungmunnithum *et al.,* 2018). Because of their nutraceutical properties, polyphenol-enriched by-products have been widely used in bakery foods. Incorporating polyphenols in bakery foods can increase antioxidant activity and scavenge foodborne toxins in baked foods (Ou *et al.,* 2019). In our previous study, the total polyphenol content in the MI-G4 variety was found to be 96 mg GAE g^{-1} on a wet basis (Satish and Urooj, 2019) and in turmeric, found to be 21.95 mg GAE g^{-1} on a dry basis (Lim *et al.,* 2017). In the present study, the total polyphenol in the MI-B and TM-B were found to be 2.01 mg GAE g^{-1} and 1.54 mg GAE g^{-1} on a dry weight basis. There was a huge loss in total polyphenol content in fortified bread compared to the raw extract of MI and raw turmeric powder. This observation explains that the baking process leads to a breakdown of polyphenols and results in the loss of polyphenols. The results agree with earlier reports that phenolics are quite heat unstable and reactive compounds under the baking process might have resulted in some heat damage to phenolic compounds, which caused to loss of some phenolic content (Cheynier, 2005). The total antioxidant activities (TAA) determined by DPPH of bread samples were expressed as mg Trolox equivalent per 100 g dried bread sample (Table 2). MI-B showed significantly higher antioxidant activities followed by TM-B > control bread. However, despite the substantial loss of total phenol content during the baking process, bread containing MI extract showed significantly higher total phenolic content with potent antioxidant capacity when compared with the control and turmeric-incorporated bread. It can be explained by the presence of heat-stable polyphenols, resulting in good antioxidant capacity. Thus, MI extract is a recommended source for developing nutraceutical bread, which can render health benefits to the human body.

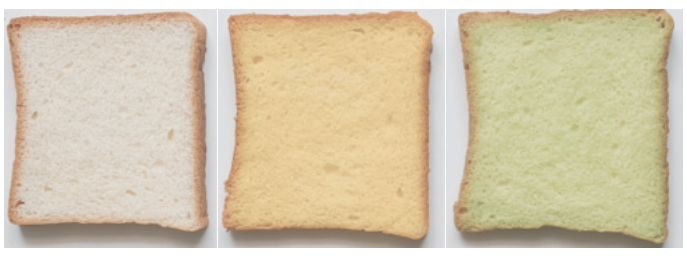

Fig. 1. Cross-sections of the prepared bread samples. Control, Turmeric bread (5 %) and *M. indica* (0.5 %)

Table 2. Total phenolic content and antioxidant capacity of fortified bread samples

Bread samples	Total phenolic (mg GAE g^{-1})	DPPH (mg Trolox/100 g DW)
Control	3.2±1.2[c]	8.3±2.1[c]
TM-B	154±4.9[b]	281±9.3[b]
MI-B	201±3.7[a]	314±11.3[a]

TM-B: 5 % Turmeric bread, MI-B: 0.5 % *M. indica* extract bread. Means with different letter superscripts within the same column are significantly different ($P < 0.05$).

Caramelization, the Maillard reaction, and lipid peroxidation can yield dicarbonyl compounds (glyoxal and methylglyoxal) while baking food products such as cake, bread, cookies, *etc.* The formation of high reactive carbonyl species (RCS) poses health risks such as pathological events and carcinogenicity (Arribas-Lorenzo and Morales, 2010). Cookies are also high in dietary AGEs, which are implicated in the development of oxidative stress and inflammation-related disorders and can be made with dicarbonyls as a precursor (Zhang *et al.,* 2014). It has been reported that polyphenols could scavenge RCS generated in baked foods (Ou *et al.,* 2019; Arribas-Lorenzo and Morales, 2010). In this context, the present work was undertaken to validate the inhibition of toxic aldehydes produced in baked fortified bread samples. MI-B was most effective against inhibition of AGEs fluorescent formation (31 % inhibition) compared to TM-B (22 % inhibition), as shown in Fig. 2. Our result suggests that the presence of polyphenols with antioxidant activities in MI extract may contribute to the reduction of total AGEs formation in MI fortified bread. Zhang *et al.* (2014) reported that the antioxidant activity in terms of free radical scavenging capacity and the inhibition of total AGEs formation showed a positive correlation. This result also corroborates with our previous study, and the MI-G4 extract significantly attenuated the oxidation of glucose, protein glycation, aggregation, crosslinking and formation of AGEs fluorescence which may be due to the presence of apigenin compound (Satish and Urooj, 2019). In general, thermal processing is one of the most used methods for food processing. A series of chemical reactions occur during heating and these reactions play a significant role in determining organoleptic qualities such as colour and flavour, nutritional value and safety of processed foods. Further, these compounds have various biological activities; some are shown to be potential antioxidative and chemopreventive agents.

In contrast, some harmful toxic compounds, such as glyoxal, methylglyoxal acrylamide and heterocyclic amines give rise to the formation of advanced glycation endproducts, which might pose significant health risks for human beings in the long term. In our current study, the results showed that bread with the addition of MI extract had stronger antioxidant activity than that of control bread, greatly enhanced the total antioxidant capacity of bread, and simultaneously decreased the level of total AGEs formation. These data indicated that MI extract fortification might be a promising way to produce functional bread that helps consumers with relatively lower AGEs-associated disorders.

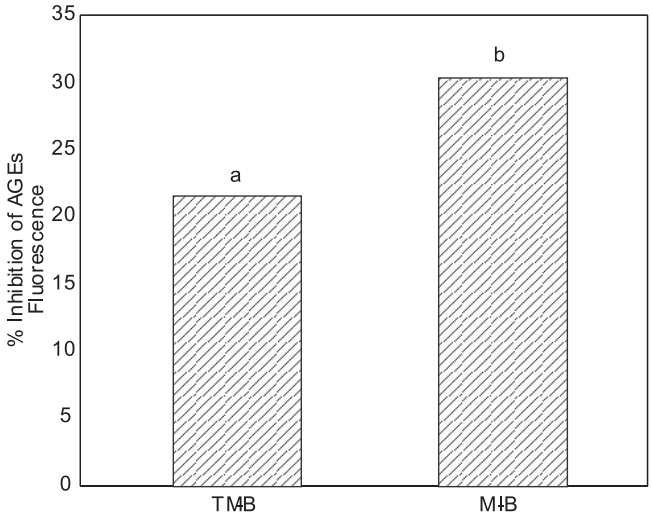

Fig. 2. Inhibitory effects of fortified bread samples on the formation of total fluorescent AGEs. Results were expressed as inhibition percentage compared with control group (without fortification). Data not contained the same letter demonstrated the difference was significant (P <0.05). TM-B: 5 % Turmeric bread, MI-B: 0.5 % *M. indica* extract bread

The incorporation of MI extract into bread significantly increased the bread's antioxidant capacity and total phenolic content and also possessed potent antiglycation activity by inhibiting the formation of total AGEs. However, before the commercialization of MI-B, future studies need to focus on the complete toxicological evaluation of newly derived compounds from the food system during the baking process.

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