

# ANTIHYPERTENSIVE PROPERTIES OF AQUEOUS EXTRACTS OF VEGETABLE LEAF-FORTIFIED BREAD AFTER ORAL ADMINISTRATION TO SPONTANEOUSLY HYPERTENSIVE RATS

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## Original article

**Antihypertensive properties of aqueous extracts of vegetable leaf-fortified bread after oral administration to spontaneously hypertensive rats**Adeola M. Alashi,<sup>1</sup>  Kehinde A. Taiwo,<sup>2</sup> Durodoluwa Oyedele,<sup>3</sup> Odunayo C. Adebooye<sup>4</sup> & Rotimi E. Aluko<sup>1\*</sup> 

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**Summary** This study investigated the potential cardiovascular health benefits of leavened bread produced from wheat flour that contained 1%, 2% and 3% additions of leafy vegetable powders obtained from *Amaranthus viridis* (AO), *Solanum macrocarpon* (SM) or *Telfairia occidentalis* (TO). Dried breads were extracted with water at 60 °C followed by analysis for total polyphenolic content (TPC), as well as *in vitro* inhibitions of angiotensin-converting enzyme and renin activities. HPLC analysis of the bread extracts indicated the presence of mainly rutin, gallic acid, myricetin and caffeic acid. TPC of the vegetable-fortified breads was significantly ( $P < 0.05$ ) higher (5.8–7.6 mg gallic acid equivalent, GAE/g) than that of control bread (5.5 mg GAE/g). Oral administration of 100 mg dried extract/kg body weight to spontaneously hypertensive rats led to reductions (up to 42 mmHg) in systolic, diastolic and mean arterial blood pressure in comparison with 20 mmHg for the control bread.

**Keywords** Bread, high blood pressure, leafy vegetables, oral gavage, polyphenols, spontaneously hypertensive rats, telemetry.

**Introduction**

Baked products, especially breads, are consumed worldwide as a major staple food for most people, with consumption ranging from one to three times daily depending on social factors and the type of bread. Therefore, baked foods could provide bioactives or be used as an avenue for the addition of various bioactive components with acceptable outcomes once the formulation is acceptable on the plate (Hayta & Gamze, 2011). For example, a regular white bread diet was shown to reduce hypercholesterolaemia in mice after a 6-week feeding period (Pozzo *et al.*, 2015). Several studies have also fortified wheat bread to improve its nutritional benefits by changing the composition of flour and/or its bioactivity through addition of ingredients such as vitamins, minerals and phytochemicals (Peng *et al.*, 2010; Jensen *et al.*, 2011; Altunkaya *et al.*, 2013). These additions can improve processing ability or alter the texture, taste and appearance and may also have negative effect on sensory attributes of the end

product, while also improving health benefits (Altunkaya *et al.*, 2013).

High blood pressure or hypertension (the pathological condition) is a prevalent ailment that affects both males and females worldwide with a huge part of developing countries, especially the African populace having the highest prevalence (NCD, 2017). Due to the high cost of antihypertensive drugs in developing countries, a plausible approach to reducing hypertension-related fatalities is the use of nutrient-fortified foods. Even in developed countries where antihypertensive drugs are highly accessible, the occurrence of negative side effects such as dry cough, oedema and erectile dysfunction also provides opportunity for intervention with fortified foods (Gunkel *et al.*, 1996; Flack *et al.*, 1997; Tenenbaum *et al.*, 2000). Human blood pressure is regulated mainly by the renin-angiotensin system (RAS) whereby renin converts angiotensinogen to inactive angiotensin-I, which is then acted upon by angiotensin-converting enzyme (ACE) to form angiotensin-II, a powerful vasopressor peptide (Santos *et al.*, 2012; Aluko, 2015). Under conditions of excessive activities of RAS such as disease,

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old age or nutritional imbalance, there is over-production of angiotensin-II, which prevents adequate relaxation of blood vessels and leads to the development of high blood pressure (Aluko, 2015). Therefore, compounds (mostly drugs) that reduce renin and ACE activities have been traditionally used to enhance blood vessel relaxation and prevent or treat high blood pressure. However, the associated negative side effects of drugs have led to the development of natural compounds that can prevent or provide relief from high blood pressure. One group of such natural compounds is the polyphenols, which are highly abundant in plant products and are known inhibitors of the RAS components, especially ACE (Actis-Goretta *et al.*, 2006; Takahashi *et al.*, 2008; Xie & Zhang, 2012; Shaw *et al.*, 2017). For example, oral administration of a polyphenolic-rich fruit beverage to spontaneously hypertensive rats (SHR) resulted in decreased blood pressure (Gunathilake *et al.*, 2013). Chokeberry juice and its polyphenols at a dose of 50 mg kg<sup>-1</sup> body weight/day were also reported to produce substantial reductions in SHR blood pressure (Hellstrom *et al.*, 2010). However, a recent report on a leaf (*Ocimum gratissimum*) polyphenol extract indicated decreased blood pressure only at a high dose of 500 mg kg<sup>-1</sup> body weight of SHR (Shaw *et al.*, 2017).

Leafy vegetables are very rich sources of phytochemicals, especially polyphenols that can contribute to improved human health. This is because even though these phytochemicals have few or zero caloric values, they possess bioactive properties that have been linked to reduced risks of chronic diseases such as antioxidative, antihypertensive, anticancer, anti-bactericidal and antiviral properties at low concentrations (Kayode & Kayode, 2011; Sreeramulu *et al.*, 2013; Gawlik-Dziki *et al.*, 2014; Oboh *et al.*, 2016; Shaw *et al.*, 2017). Several works have focused on the extraction of polyphenols from leafy vegetables, including determination of the extract bioactive properties while others have studied the prevention of lipid peroxidation and other antioxidant properties (Kayode & Kayode, 2011; Segovia Gómez & Almajano Pablos, 2016; Shaw *et al.*, 2017). However, research that focuses on both the *in vitro* and *in vivo* activities of polyphenol-fortified food systems is limited, especially with respect to blood pressure reduction. Previous works have shown the potential use of vegetables to enhance bioactive properties of bread. For example, phenolic contents as well as antioxidant properties were shown to be higher in vegetable-enriched wheat breads than plain bread (Świeca *et al.*, 2013, 2014; Ranawana *et al.*, 2016a,b).

Therefore, our study investigated the high blood pressure-lowering ability of bread fortified with vegetable leaf powder obtained from three most

commonly consumed green leafy vegetables in West Africa: *Amaranthus viridis* (AV) or amaranth; *Solanum macrocarpon* (SM) or African eggplant; and *Telfaria occidentalis* (TO) or fluted pumpkin. These leaves are used by mostly all ethnic groups and regions in West Africa for various dishes and side dishes. These vegetables have both medicinal and nutritional properties because they have been found to contain phenols such as rutin, gallic acid, catechin, caffeic acid and quercetin, which can contribute to improved human health (Katerere *et al.*, 2012; Oboh *et al.*, 2016). The main aim of this work was to determine the blood pressure-lowering effects of vegetable leaf-fortified leavened bread samples following oral administration of their aqueous extracts to SHR. The ability of the aqueous extracts to inhibit *in vitro* activities of renin and ACE activities was determined to evaluate the potential influence on the RAS.

## Materials and methods

### Materials

Rabbit lung ACE, *N*-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG), captopril and polyphenol standards were purchased from Sigma Chemicals (St. Louis, MO, USA). Human recombinant renin inhibitor screening assay kit was purchased from Cayman (Cayman Chemical, Ann Arbor, MI, USA). All other reagents were of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada).

### Preparation of vegetable leaf-fortified leavened bread samples

Vegetable leaves were harvested from the MicroVeg Project 107983 site located at the Teaching and Research Farm, Obafemi Awolowo University, Ile-Ife, Nigeria. Leaves were dried and incorporated into wheat flour leavened bread formulations as previously described (Famuwagun *et al.*, 2016). Briefly, freshly harvested leaves were cut into small pieces, dried in a hot air cabinet at ~60 °C for 8 h and then milled into powder in a Marlex Excella dry mill (Marlex Appliances PVT, Daman, India). The dried leaves were substituted for enriched wheat flour at 1%, 2% and 3% (w/w) using 200 g batches. The straight-dough method was used to mix the ingredients as follows: 200 g of composite flour blends (wheat flour + vegetable powder), yeast (6 g), salt (4 g), oil (10 g), sugar (6 g) and water (120 mL). Dough preparation and baking were conducted under previously reported conditions (Famuwagun *et al.*, 2016). After cooling, the baked bread was cut into small pieces, freeze-dried and ground into a powder using a laboratory blender.

### Preparation of bread polyphenol extracts

Aqueous polyphenol extracts were prepared from dried bread powders as previously reported (Lafarga *et al.*, 2016). Briefly, ground bread powders were extracted using double distilled water at 1:20 (powder:water) ratio for 2 h at 60 °C under constant stirring. The samples were cooled to room temperature and centrifuged (8000 × *g*) to obtain the first supernatant. The residue was re-extracted with twenty volumes of water under the same conditions and then centrifuged as before to obtain a second supernatant. Both supernatants were pooled, concentrated under vacuum in a rotary evaporator, freeze-dried and stored at −20 °C.

### Determination of the total phenolic contents in bread extracts

The bread extracts and gallic acid (standard) were each mixed with 50% methanol to obtain 1 and 10 mg mL<sup>−1</sup> stock solutions, respectively, which were then passed through a 0.45 μm syringe filter to remove particulates. TPC of the bread extracts was determined according to the Folin–Ciocalteu method as previously described (Fasakin *et al.*, 2011).

### High-pressure liquid chromatography (HPLC)

The polyphenolic profile of each bread extract was determined using a 5 μm C18 analytical (250 × 4.6 mm) reverse-phase HPLC column (Phenomenex Inc., Torrance, CA, USA) fitted on a Varian 940-LC system (Agilent Technologies, Santa Clara, CA, USA). Polyphenol standards (gallic acid, catechin, rutin, myricetin and caffeic acid) were dissolved in ethanol at 0.5 while 10 mg mL<sup>−1</sup> of the dried bread extracts was prepared in 1% (v/v) acetic acid. A 100 μL aliquot of each standard or sample was injected onto the column at 37 °C. An isocratic gradient was used with 1% acetic acid as elution buffer. Elution times of the peaks obtained from the standards were compared to those of the samples to identify their polyphenol profiles.

### Angiotensin-converting enzyme and renin inhibition assays

The *in vitro* inhibition of ACE (0.5 mg mL<sup>−1</sup>) and renin (1 mg mL<sup>−1</sup>) activities was determined according to previously described methods (Alashi *et al.*, 2014). FAPPG was used as ACE substrate, and absorbance changes were recorded at 37 °C and 345 nm wavelength for 30 min at 1 min intervals. ACE activity was expressed as the change in the rate of reaction ( $\Delta A/\text{min}$ ), and inhibitory activity was calculated using the following equation:

$$\text{ACE inhibition(\%)} = \left( \frac{\text{Slope}(\Delta A/\text{min})_{(\text{blank})} - \text{Slope}(\Delta A/\text{min})_{(\text{sample})}}{\text{Slope}(\Delta A/\text{min})_{\text{blank}}} \right) * 100$$

where  $(\Delta A/\text{min})_{(\text{blank})}$  and  $(\Delta A/\text{min})_{(\text{sample})}$  are ACE activities in the absence and presence of samples, respectively.

Renin inhibition was carried out using the renin assay kit according to the manufacturer's instructions. The fluorescence intensity was measured at excitation and emission wavelengths of 340 and 490 nm, respectively for 10 min. Percentage inhibition of renin was calculated using the following equation.

$$\text{Renin inhibition(\%)} = \left( \frac{(\text{FI of blank well} - \text{FI of sample well})}{(\text{FI of blank well})} \right) * 100$$

### Blood pressure measurements

The animal study was carried out in conformity with the Canadian Council for Animal Care guidelines and according to protocols approved by the University of Manitoba Animal Care Committee. Male SHR rats at 6 weeks were purchased from Charles River Laboratories (Montreal, PQ, Canada) and implanted with telemetry sensors after 2 weeks of acclimatisation (under 12-h day and night cycle at 21 °C) with *ad libitum* access to regular chow feed and tap water. Details of the surgical implantation of sensors have been previously described by O'Keeffe *et al.* (2017). The SHR rats were allowed a 2-week recovery from the surgery before proceeding to oral administration of test agents. Oral administration of the bread extracts was conducted as previously reported (Lafarga *et al.*, 2016) with captopril (antihypertensive drug) as a positive control. The bread extracts were orally administered in a total volume of 1 mL at 100 mg kg<sup>−1</sup> rat body weight dose while captopril was 20 mg kg<sup>−1</sup> body weight. Real-time systolic and diastolic blood pressure (SBP and DBP, respectively) measurements (mmHg), mean arterial pressure (MAP) (mmHg) and heart rates (HR) in beats per min (bpm) were collected in a quiet room with each rat cage placed on the respective receiver (Model RPC-1; DSI instruments, St. Paul, MN, USA). Data were recorded continuously at 10 min intervals for 24 h using the Ponemah 6.1 data acquisition software (DSI instruments). The system was linked to an APR-1 atmospheric pressure monitor (DSI instruments), which normalises the transmitted pressure values to produce blood pressure signals independent of atmospheric pressure changes. Results are

reported as changes in values of the SBP, DBP, MAP and HR at 2, 4, 6, 8, 12 and 24 h minus their baseline measurements at time zero.

### Statistical analysis

Data are presented as mean  $\pm$  standard deviation and analysed by analysis of variance with *post hoc* evaluation carried out using Duncan's Multiple Range test to determine significant differences at  $P < 0.05$  using SPSS version 22.0.

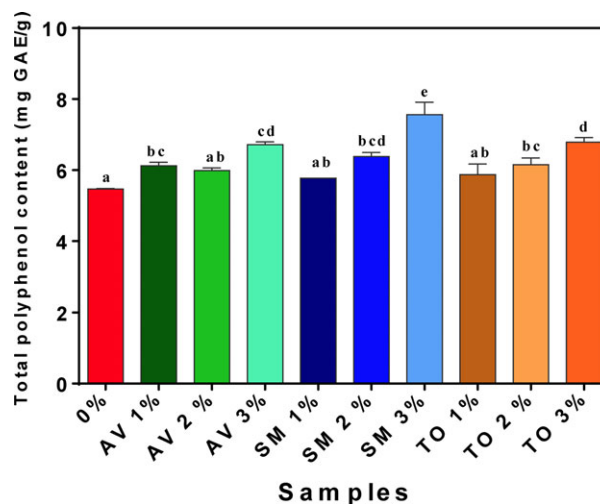
## Results

### Total polyphenolic content and polyphenol profiles

Total polyphenolic content of bread extracts was measured with respect to the mg GAE/g polyphenol-fortified bread on a dry weight basis. Figure 1 shows that bread fortification with the leafy vegetables resulted in significant ( $P < 0.05$ ) increases in TPC depending on the inclusion level. All the bread samples fortified with 3% vegetables had significantly higher TPC levels than the control white bread. However, incorporation of 2% AV, 1% TO and 1% SM did not produce significant changes in TPC when compared to the control white bread. The polyphenol profiles of the fortified bread extracts showed similar levels with gallic acid, catechin, rutin, myricetin and caffeic acid as the main identifiable compounds (Fig. 2). However, the control bread did not contain a measurable level of the peak that eluted at 35 min, which suggests that the compound in this peak originated mainly from the vegetable leaves. As expected, the profile did not change when different levels of the leaf extracts were incorporated into the bread samples.

### *In vitro* angiotensin-converting enzyme and renin inhibitions

Figure 3a and b shows that the control- and vegetable-fortified bread extracts inhibited ACE and renin activities at 0.5 and 1 mg mL<sup>-1</sup>, respectively, although to different extents. The control bread extract had significantly ( $P < 0.05$ ) highest ACE activity inhibition (23.9%), which could have been due primarily to the peptides that were released from yeast enzyme-dependent hydrolysis of wheat proteins during dough fermentation (Fig. 3a). The ACE-inhibitory activity for AV-fortified bread samples increased significantly and was dose-dependent (10.6%–15.6%) from 1% to 3%. However, ACE inhibition by SM- and TO-fortified bread extracts decreased (15.5%–6.8% and 18.0%–4.6%, respectively) as level of fortification increased from 1% to 3%. Figure 3b shows weak renin inhibition by control (5.9%) and TO (5.0%–7.7%) bread

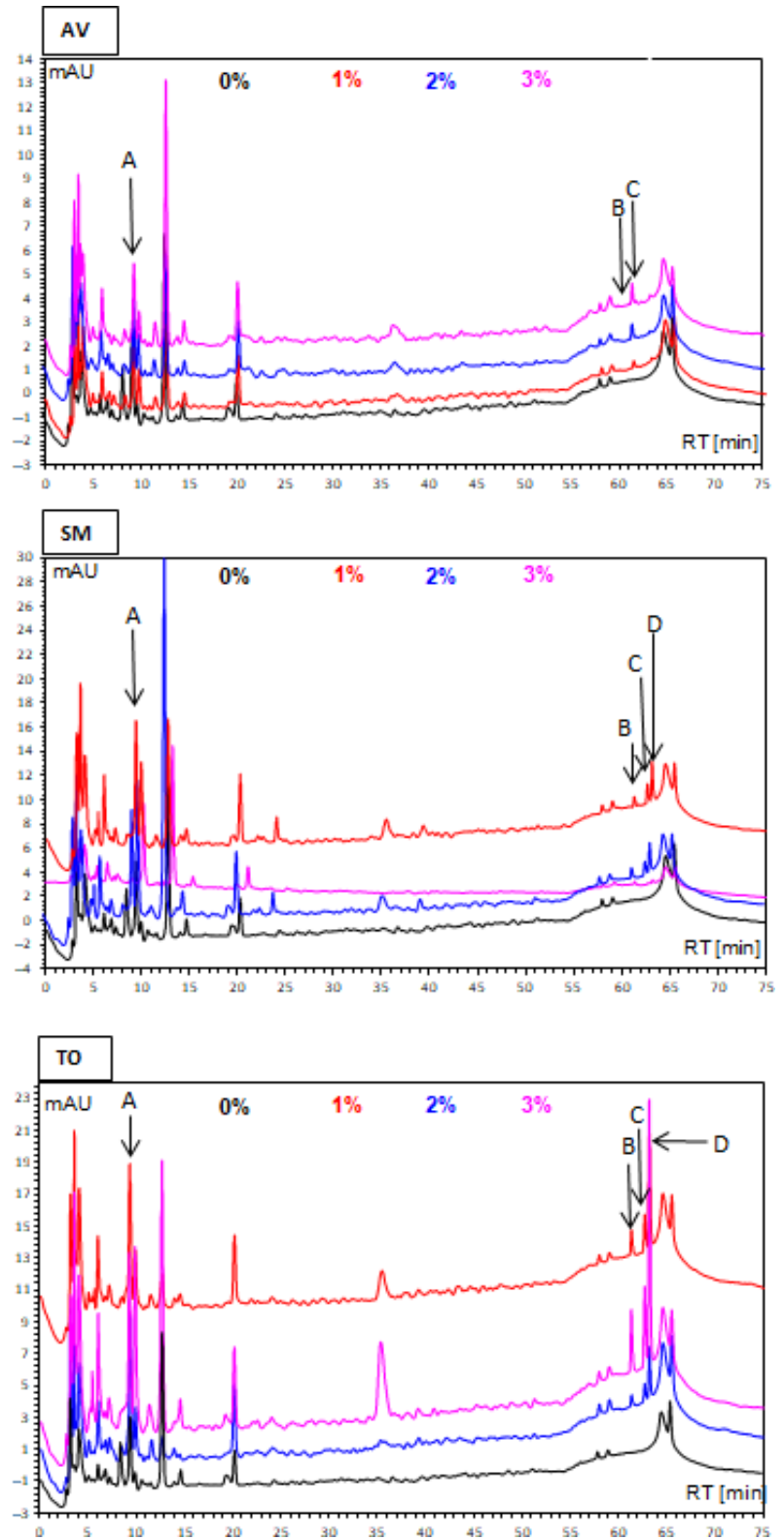


**Figure 1** Total phenolic content (mg gallic acid equivalent, GAE/g dry bread extract) of aqueous extracts of control bread (0%) or bread fortified (1%–3%) with *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) leaves. Data are expressed as mean  $\pm$  standard deviation. Bars having the same letter are not significantly different ( $P < 0.05$ ). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

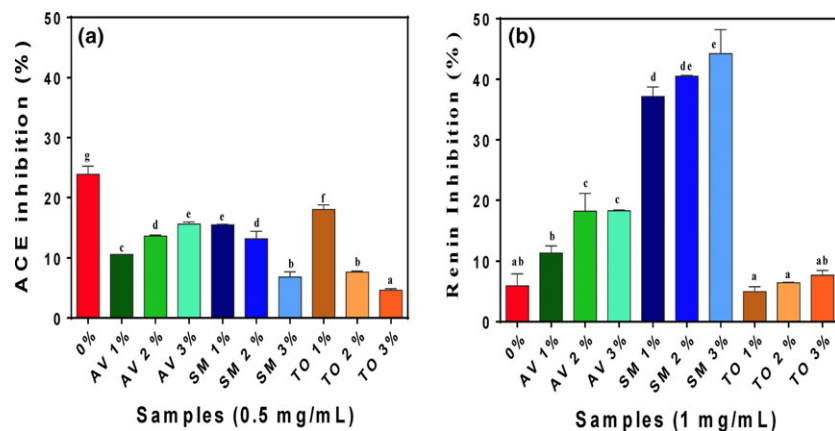
extracts, which suggest minimal ability of wheat polyphenols to interact with the enzyme protein. In contrast, renin inhibitions by AV (11.3%–18.3%)- and SM (37.2–44.2)-fortified bread extracts were significantly ( $P < 0.05$ ) higher than those of the control and TO-fortified breads.

### Systolic blood pressure and diastolic blood pressure

The ability to reduce SBP is a critical feature of anti-hypertensive agents and the three vegetable extracts were very effective in this respect (Fig. 4a). The control bread (0%) extract showed a fairly consistent (approx. –20 mmHg) SBP-lowering effect over 8 h while at 12 and 24 h, there were no significant ( $P < 0.05$ ) reductions in SBP by the control bread extract. In contrast, SBP reductions by the vegetable-fortified breads were significantly ( $P < 0.05$ ) higher especially at the 2% and 3% inclusion levels compared with the control bread. The 1% and 2% AV-fortified bread samples did not produce significantly ( $P > 0.05$ ) different SBP reductions compared with the control bread. However, the 3% AV-fortified bread produced significantly ( $P < 0.05$ ) different SBP reductions with the highest value of –42 mmHg after 4 and 6 h. For SM-fortified bread samples, only the 2% had significant ( $P < 0.05$ ) SBP-lowering effect (–42 mmHg at 4 and 6 h) that was different from that of the control bread but similar to the 3% AV-fortified bread. The 2% TO-fortified bread with up to –37 mmHg after 6 h was also



**Figure 2** HPLC profile of aqueous extracts of control bread (0%) or bread fortified (1%–3%) with *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) leaves. The different alphabets indicate the presence of the following polyphenols: A, gallic acid; B, rutin; C, myricetin; and D, caffeic acid. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Figure 3** Inhibition of *in vitro* activities of ACE (a) and renin (b) by aqueous extracts (0.5 and 1.0 mg mL<sup>-1</sup>, respectively) of control bread (0%) or bread fortified (1%–3%) with *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) leaves. Data are expressed as mean  $\pm$  standard deviation. Bars with the same letter are not significantly different ( $P < 0.05$ ). ACE, angiotensin-converting enzyme. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

significantly ( $P < 0.05$ ) better than the 1% and 3% in reducing SBP. The DBP also showed significant ( $P < 0.05$ ) reductions at same vegetable levels (3% for AV and 2% for SM and TO) that produced maximum SBP-lowering effects (Fig. 4b). The maximum DBP reduction for the control bread was  $-20$  mmHg and was fairly consistent for 8 h and became very weak at 12 and 24 h, which is similar to the SBP effects. The 2% TO-fortified bread produced the strongest DBP reductions with maximum of  $-42$  mmHg at 6 and 8 h compared with  $-31$  mmHg for 3% AV and 2% SM with  $-31$  mmHg. The 3% AV or 2% SM and TO also produced significantly ( $P < 0.05$ ) long-lasting DBP reductions with up to  $-27$  mmHg after 24 h when compared to the control bread ( $-4$  mmHg).

#### Mean arterial pressure

The MAP showed similar patterns as the SBP and DBP but with higher reductions and similar values at 4–12 h for the 1% AV and 3% AV-fortified bread samples (Fig. 5). The control bread showed very weak MAP reduction after 2 h ( $-7$  mmHg), increased to a maximum at 6 h ( $-24$  mmHg) but with decreased effect at 12 h ( $-7$  mmHg) and 24 h ( $-5$  mmHg). In comparison with the control bread, the vegetable-fortified bread samples had significantly ( $P < 0.05$ ) higher MAP values, especially the 1% ( $-40$  mmHg at 4 h) and 3% AV ( $-38$  at 4 h). The 2% SM and TO-fortified breads were also more potent than the control bread as MAP-reducing agents with maximum values of  $-37$  mmHg at 6 h and  $-42$  mmHg at 8 h, respectively.

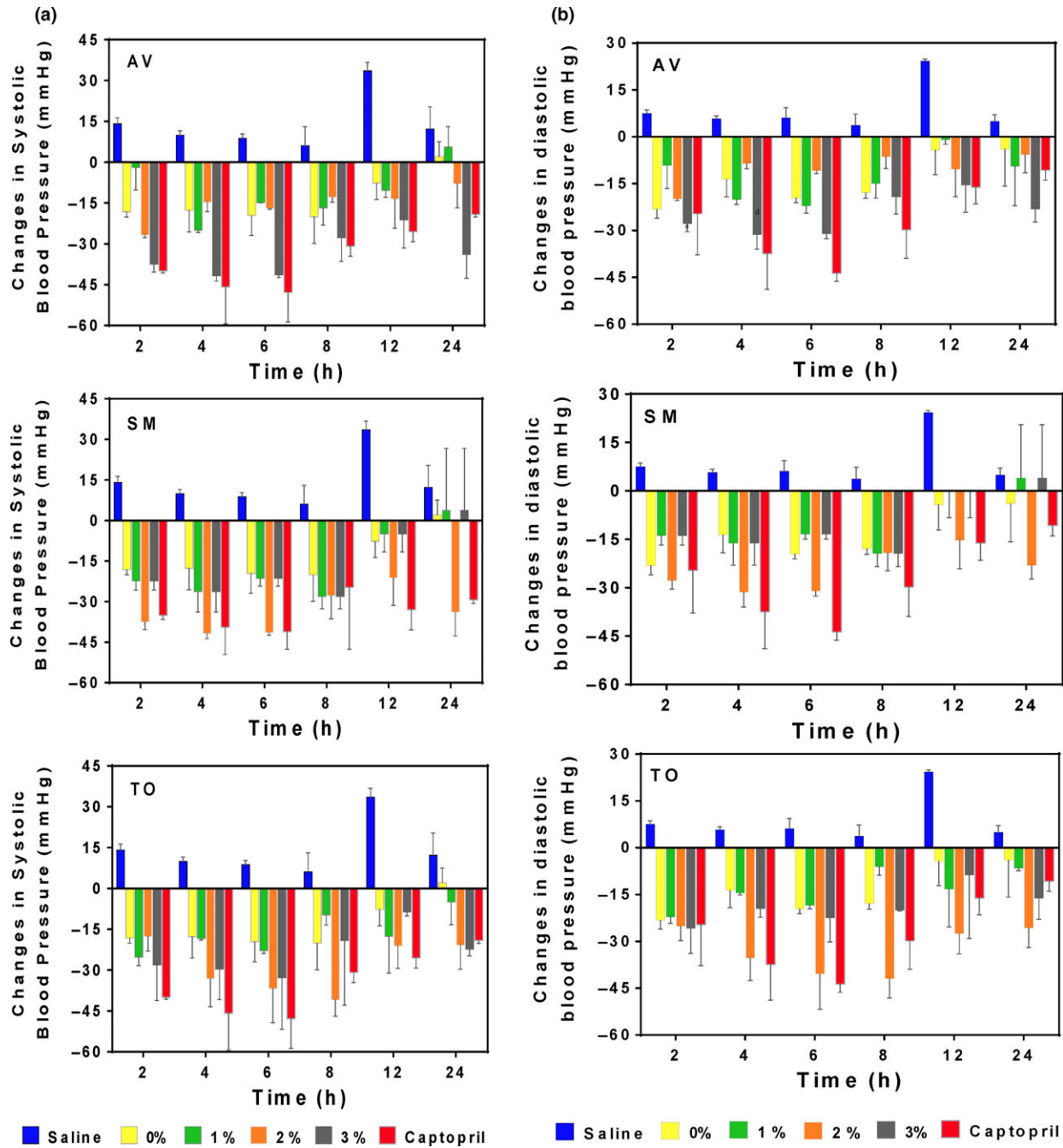
#### Heart rate

The average resting heart rates of the rats range from 315 to 330 bpm prior to oral administration of test samples. Unlike blood pressure, vegetable-fortified bread was not as effective as captopril ( $-166$  bpm) in

reducing heart rate (Fig. 6). However, the 3% AV-fortified bread and 2% SM-fortified bread extracts had significantly higher heart rate reductions with maximum  $-102$  (bpm) at 6 h. This value is higher ( $P < 0.05$ ) than the  $-43$  bpm obtained for the control bread. The TO-fortified bread extract produced a maximum heart rate reduction of  $-66$  bpm, which was not significantly different from the control bread. But the  $-66$  bpm effect of TO-fortified bread is lower than the  $-102$  bpm obtained for the 3% AV-fortified bread and 2% SM-fortified bread.

#### Discussion

In this work, aqueous extracts were used because initial extraction of the dried bread samples with organic solvents or aqueous organic solvent mixtures produced very insoluble materials. These organic extracts did not dissolve properly in the aqueous assay media used for various experimental protocols. Vegetable fortification of wheat flour has been previously demonstrated by various researchers as a veritable means of enhancing the nutritional quality of bread samples (Gawlik-Dziki *et al.*, 2014; Świeca *et al.*, 2014; Ranawana *et al.*, 2016a). For example, breads fortified with different vegetables such as carrot, tomato, beetroot, and broccoli (Ranawana *et al.*, 2016a) or onion skin (Świeca *et al.*, 2013) had higher TPC content than plain bread. Specifically, the addition of 4% (w/w) onion skin to wheat flour produced breads that contained 6.5 mg GAE/g TPC when compared to  $\sim 4.0$  mg GAE/g for the control (Świeca *et al.*, 2013). Therefore, results from this work are consistent with previously reported increases in TPC of vegetable-fortified bread. The TPC values obtained in this work ranged from 5.5 mg GAE/g in the control bread to a maximum of 7.6 mg GAE/g in the bread fortified with 3% SM, which are higher than the data reported for onion skin and broccoli sprout-fortified breads. The differences in TPC of control bread could be due to the wheat

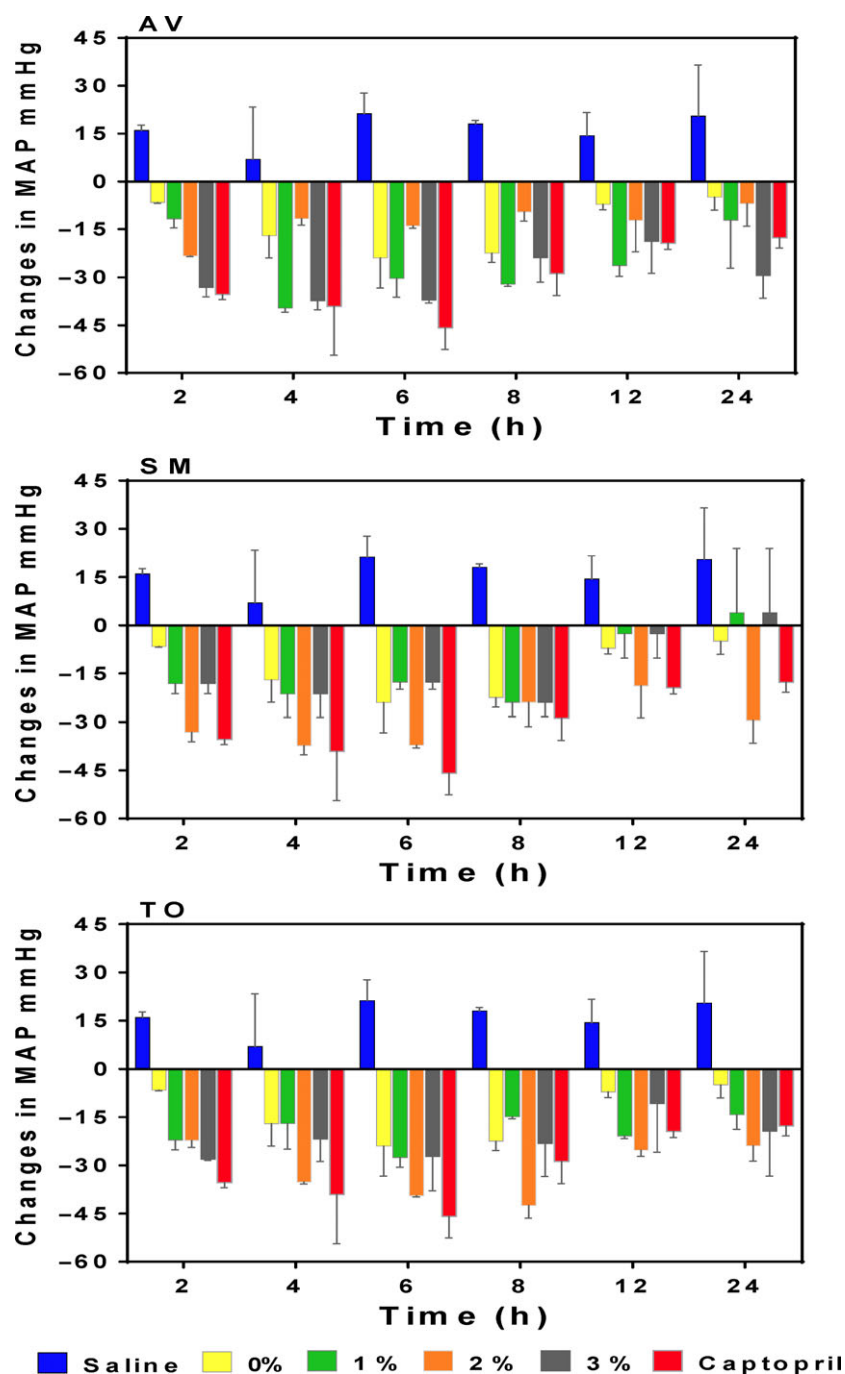


**Figure 4** Changes in systolic blood pressure (a) and diastolic blood pressure (b) of spontaneously hypertensive rats after oral gavage ( $100 \text{ mg kg}^{-1}$  body weight) using aqueous extracts from control bread (0%) or bread fortified (1%–3%) with *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) leaves. Data are expressed as means  $\pm$  standard deviation. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

variety used in each study while the higher TPC values of fortified breads in this work suggest that the leaves contained higher polyphenol contents than the onion skin and broccoli sprouts. Wheat flour fortification

with quinoa leaves (1%–5%, w/w) was also shown to dose-dependently increase bread TPC content (Świeca *et al.*, 2014). However, the HPLC chromatogram shows that there were only minor changes in the



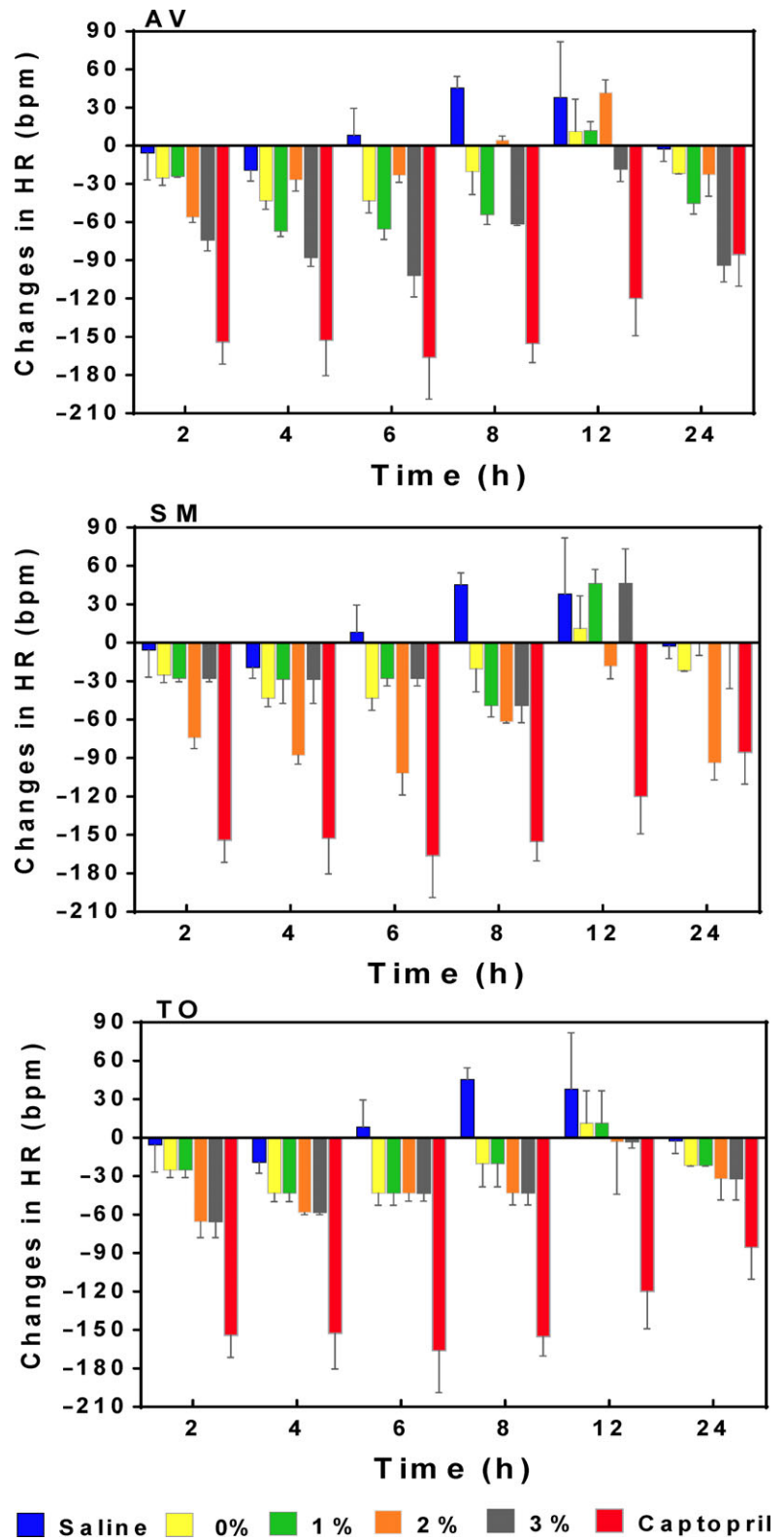


**Figure 5** Changes in mean arterial pressure (MAP) of spontaneously hypertensive rats after oral gavage ( $100 \text{ mg kg}^{-1}$  body weight) using aqueous extracts from control bread (0%) or bread fortified (1%–3%) with *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) leaves. Data are expressed as means  $\pm$  standard deviation. MAP, mean arterial pressure. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

polyphenol profile when different levels of the leaf extracts were incorporated into the bread samples. Apart from catechin, a previous work has also shown the presence of gallic acid, rutin, myricetin and caffeic acid in TO and AV leaves (Oboh *et al.*, 2016).

The human RAS that regulates blood pressure is controlled mainly by the protease activities of ACE

and renin; hence their *in vitro* inhibitions are typically used to measure potential antihypertensive effects (Alashi *et al.*, 2014; Girgih *et al.*, 2016). The ACE-inhibitory activity of the control bread (24%) in this work is higher than the  $\sim 10\%$  ACE inhibition reported for plain wheat bread (Peñas *et al.*, 2015). The difference may be due to variation in the type of proteins present



**Figure 6** Changes in heart rate (measured as beats per minute, bpm) of spontaneously hypertensive rats after oral gavage (100 mg kg<sup>-1</sup> body weight) using aqueous extracts from control bread (0%) or bread fortified (1%–3%) with *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) leaves. Data are expressed as means ± standard deviation. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

in the wheat flours or the proteolytic activity of the yeasts could have differed. The ACE-inhibitory activity for AV-fortified bread samples increased significantly and was dose-dependent (10.6%–15.6%) from 1% to 3%. However, ACE inhibition by SM- and TO-fortified bread extracts decreased (15.5%–6.8% and 18.0%–4.6%, respectively) as the level of fortification increased from 1% to 3%. The exact reasons for these differences are not clear but it is possible that at 1% AV fortification, polyphenols bind to ACE protein in such a way that more polyphenolic compounds can still bind to increase inhibitory potency at 2 and 3% levels of AV in the bread. In contrast, at the 1% SM and TO inclusion level, there was maximum binding to ACE protein; as the level increased to 2 and 3%, polyphenol–polyphenol interactions may be stronger than polyphenol–ACE interactions, hence reduced ACE-inhibitory activity. The lower ACE inhibition by vegetable-fortified breads may have been due polyphenol interactions with peptides, which led to weak peptide binding to the ACE protein when compared to the control bread. It is also possible that the polyphenols bind to proteases released by yeast during fermentation, which reduced the release of ACE-inhibitory peptides from the wheat proteins. This protease inhibition would have increased as the level of polyphenol addition was raised from 1% to 3%, hence the associated decreases in measured ACE-inhibitory activity. ACE-inhibitory activities of polyphenol-fortified breads are scarce but previous works have also shown various inhibitory activities of polyphenol extracts from chokeberry (Hellstrom *et al.*, 2010), *Ocimum gratissimum* leaves (Shaw *et al.*, 2017) and other leafy vegetables (Obloh *et al.*, 2016).

The renin inhibition data suggest that at 1% bread fortification, the AV and SM polyphenols were bound weakly to renin protein but increased fortification to 2% and 3% provided more polyphenols that could bind to renin with resultant increases in inhibitory potency. While the inhibitory pattern for AV was similar for both ACE and renin, the inverse relationship for SM suggests a different mechanism of interactions. Reports of renin inhibition by polyphenols are scarce but a previous work has also shown dose-dependent activities by soybean saponins although at lower concentrations (5–80  $\mu\text{g mL}^{-1}$ ) than the 1 mg  $\text{mL}^{-1}$  used for the bread extracts (Takahashi *et al.*, 2008). The stronger activity of the soybean saponins could be attributed to its higher purity (Takahashi *et al.*, 2008) when compared to the crude bread extracts.

The blood pressure-reducing ability of the bread extracts suggests their potential use as antihypertensive functional food products. The 100 mg dried extract/kg SHR body weight dose translates to  $\sim 1.14$  g dried extract/day for a 70 kg human being based on the rat to human dose conversion factor (Reagan-Shaw *et al.*,

2007). This effective blood pressure-reducing dose can readily be achieved through daily consumption of a few slices of vegetable-fortified bread and could be a nutritionally desirable means of maintaining a normal blood pressure. MAP reflects the average blood pressure in a cardiac cycle and lower levels are beneficial in promoting cardiovascular health. The long-lasting (up to 24 h) reductions in MAP and SBP of the vegetable-fortified breads suggest that the polyphenols were not rapidly cleared from the blood, which could provide effective daily management of blood pressure. The values obtained in this work are similar to the  $-20$  mmHg SBP reductions reported after oral administration of 50 mg chokeberry polyphenols/kg SHR body weight (Hellstrom *et al.*, 2010). This is because 100 mg of the vegetable-enriched breads produced about twice the SBP reduction reported for 50 mg chokeberry polyphenols. But the results obtained in this work show stronger SBP-reducing effects of the vegetable-fortified breads when compared to oral administration of 500 mg  $\text{kg}^{-1}$  SHR body weight of a vegetable leaf polyphenol extract that lowered SBP by only 8 mmHg (Shaw *et al.*, 2017). The vegetable-fortified breads also have stronger SBP-reducing effects than lingonberry juice, which produced 26 mmHg reductions after oral administration to SHR (Kivimaki *et al.*, 2013). It is possible that the stronger SBP-reducing effects of the vegetable-enriched breads may be due to synergistic interactions of polyphenols with bioactive peptides that are present in leavened bread (Zhao *et al.*, 2013; Peñas *et al.*, 2015) when compared to the vegetable leaf extract or lingonberry juice that contained only polyphenols. The higher SBP-reducing ability of the vegetable-fortified bread could also be as a result of synergistic interactions between vegetable polyphenols and wheat polyphenols. For example, simultaneous consumption of tea and soy was found to lead to greater antioxidant property than when consumed alone (Bertipaglia de Santana *et al.*, 2008). More importantly, both the 2% SM-fortified and 3% AV-fortified bread extracts produced significant ( $P < 0.05$ ) long-lasting effects with  $-34$  mmHg SBP reductions after 24 h when compared to  $+3$  mmHg for the control bread. The results suggest that vegetable incorporation into regular white leavened bread enhanced bioactive properties with respect to blood pressure reduction. The 1% fortification was not effective for all the vegetable samples, while the 2% AV produced weak SBP-lowering effects, which indicate insufficient amount of bioactive agents at these levels. It is interesting to note that SBP-lowering ability of SM and TO was strongest at the 2% inclusion level and actually became weak at the 3% level. Reduction in SBP-lowering effect at the 3% SM and TO in comparison with 2% may be due to polyphenol aggregation at the high concentration (Plumb *et al.*, 1998; Saint-Cricq de Gaulejac *et al.*, 1999) during intestinal transit, which reduced bioavailability

(absorption). This is because previous works have suggested that polyphenol absorption from the gastrointestinal tract is highly dependent on size with smaller units favored over bigger aggregates (Gonthier *et al.*, 2003; Manach *et al.*, 2005).

The DBP data are similar to previous works that have shown reductions in DBP of SHR after consumption of 50 mg chokeberry/kg body weight (Hellstrom *et al.*, 2010) and human volunteers who consumed 100 g of red beetroot-enriched bread (Hobbs *et al.*, 2012). In contrast, oral administration of 500 mg vegetable leaf (*O. gratissimum*) polyphenol extract/kg SHR body weight did not produce any significant DBP reduction (Shaw *et al.*, 2017). The difference may be due to synergistic interactions between vegetable polyphenols and wheat polyphenols as discussed above. However, the fact that in addition to polyphenols, the bread extracts also contain proteins may have led to the production of bioactive peptides during baking and gastrointestinal digestion in the rat, hence higher DBP-reducing effects than the *O. gratissimum* polyphenol extract. This is based on previous works that showed the presence of ACE-inhibitory peptides in leavened wheat bread (Zhao *et al.*, 2013; Peñas *et al.*, 2015). Reduced DBP-reducing effect for the 3% SM and TO-fortified bread could have been due to polyphenol aggregation within the gastrointestinal tract, which led to reduced bioavailability as discussed above. Reductions in heart rate also suggest additional cardiovascular benefits of the vegetable extracts. For example, oral administration of lingonberry juice led to only 26 bpm decreases in heart rate (Kivimaki *et al.*, 2013), which is less than the results obtained in this work. In contrast, oral administration of 500 mg kg<sup>-1</sup> body weight of *O. gratissimum* polyphenol extract produced no significant change in SHR heart rate (Shaw *et al.*, 2017). The potential release of bioactive peptides from bread proteins during yeast fermentation (Zhao *et al.*, 2013; Peñas *et al.*, 2015) in synergy with polyphenols may have contributed to the stronger heart rate-reducing ability of the vegetable-fortified breads. As the control bread also contained proteins, the results suggest that effects of the vegetable polyphenols alone or in synergy with bioactive peptides were stronger than similar interactions between wheat polyphenols and bioactive peptides. The results are consistent with a recent report that showed oral administration of an anthocyanin-rich plum juice to human volunteers resulted in significant reductions in heart rate within a 24 h test period (Igwe *et al.*, 2017).

## Conclusions

This work showed that fortification of wheat bread with vegetable leaves can enhance polyphenolic content and lead to improved cardiovascular health. Benefit of fortification was confirmed with the higher TPC of the test

breads when compared to the control bread. The ability of a heat-processed food like bread to retain its bioactivity *in vivo* indicates that the vegetable polyphenols were resistant to heat-induced structural degradation. The results showed that the control bread was not as effective as the vegetable-fortified breads in reducing important cardiovascular parameters such as blood pressure and heart rate. Therefore, the reduced blood pressure and heart rate-reducing abilities can be attributed to the presence of additional polyphenols from the leafy vegetables. Overall, the 2% SM-fortified bread produced the strongest cardiovascular benefits in terms of blood pressure and heart rate reductions. It is estimated that a 70 kg human being will need to consume approx. 10 g of the fresh vegetable-fortified bread daily to achieve the observed cardiovascular health benefits. The use of human intervention trials in the future is required to confirm the cardiovascular health benefits observed in this work.

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## Conflict of interest

Authors declare no conflict of interest.

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