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ABSTRACT

Both advanced glycation endproducts (AGEs) and advanced lipoxidation endproducts (ALEs) are implicated in many age-related chronic diseases and in protein ageing. In this study kawain, methysticin and dihydromethysticin, all belonging to the group of kavalactones, were identified as AGEs inhibitors. With IC_{50} values of 43.5 ± 1.2 μM and 45.0 ± 1.3 μM for kawain and methysticin, respectively, the compounds inhibited the *in vitro* protein glycation significantly better than aminoguanidine (IC_{50} = 231.0 ± 11.5 μM; p = 0.01), an established reference compound. Kawain and methysticin also inhibited the formation of dicarbonyl compounds, which are intermediates in the process of AGEs formation. Similarly, kawain and aminoguanidine prevented the formation of thiobarbituric reactive substances (TBARs) in both low density lipoprotein (LDL) and linoleic acid oxidation. Moreover, kawain and aminoguanidine prevented AGEs formation by chelating Fe^{3+} and Cu^{2+} two-three times better than aminoguanidine. Furthermore, kawain increased the mean life span of *Caenorhabditis elegans* exposed to high glucose. With glycation inhibiting, lipid peroxidation inhibiting, metal chelating properties, and lifespan extending ability, kavalactones show a high potential as AGEs and ALEs inhibitors.

Keywords: Advanced Glycation Endproducts (AGEs), Advanced Lipoxidation Endproducts (ALEs), kavalactones, kawain, chelation, *Caenorhabditis elegans*

List of abbreviations

AA	Ascorbic Acid
AG	Aminoguanidine
AGEs	Advanced Glycation Endproducts
ALEs	Advanced Lipoxidation Endproducts
BSA	Bovine Serum Albumin
GO	Glyoxal
KL	Kavalactones
KW	Kawain
LA	Linoleic Acid
LDL	Low Density Lipoprotein
MDA	Malondialdehyde
RCS	Reactive Carbonyl Species
ROS	Reactive Oxygen Species
TBARs	Thiobarbituric Reactive Substances

Introduction

In spite of improved treatments, age-related diseases are still leading causes of death worldwide. At the same time, it is well know that both advanced glycation endproducts (AGEs) and advanced lipoxidation endproducts (ALEs) are implicated in many age-related chronic diseases and in protein ageing. These products are associated with diabetic complications, atherosclerosis, uraemia, neurodegenerative diseases, cancer and the normal ageing processes [1–6]. The formation of AGEs / ALEs begins with the autoxidation of sugars or lipids. Further interactions with proteins will generate several intermediates including Schiff's bases, Amadori products, hydroperoxides, and carbonyl compounds and lipid peroxy- radicals, which are formed by lipid peroxidation. Furthermore, reactive oxygen species (ROS) and free metal ions have been identified as key participants in these biochemical reactions.

Kavalactones are a class of lactone compounds found mainly in *Piper methysticum* G. Forst (Kava), the aqueous extract of which plays a central role in the social life of the natives of Polynesia, Melanesia and Micronesia [7]. Similarly, they are also found in *Alpinia zerumbet*, a plant which is used to prepare a traditional food in the Okinawan islands [8]. The relaxing and calming properties of these traditional preparations have made them very popular among the Pacific islanders. Out of several kavalactones identified in Kava shrubs, kawain, 7,8-dihydrokawain, methysticin, dihydromethysticin, yangonin and desmethoxyyagonin have been studied extensively [9]. In our previous study, we have identified two major kavalactones, 5,6-dehydrokawain, and dihydro-5,6-dehydrokawain as AGEs inhibitors [10]. Therefore in the present work the AGEs inhibition by three other kavalactones was investigated and the mode of action unraveled.

Although the AGEs and ALEs follow different pathways, they are so interrelated that both reactions should be considered simultaneously [11]. Furthermore, several natural and

synthetic AGEs inhibitors have been proposed and tested as inhibitors of ALEs formation [12]. Therefore it was investigated if kawain also has ALEs inhibition properties through low density lipoprotein (LDL) and peroxidation assays. Furthermore, both the formation of AGEs and ALEs are initiated by adventitious transition metal ions present in the biochemical systems, and therefore the chelating kinetics of kawain in the glucose-BSA model were monitored, and the metal catalyzed linoleic acid oxidation was investigated. Finally, we examined lifespan extension property of test compounds using the nematode *Caenorhabditis elegans* as an animal model. Together, this study identifies kavalactones as a novel class of compounds against AGEs and ALEs formation, which may have a future role in preventing related pathologies.

Results and Discussion

The prevention of AGEs formation was investigated by determining the fluorescence spectra of the complex formed after incubation of glucose and protein (Bovine Serum Albumin, BSA) in the presence or absence of test compounds (Fig.1). The inhibition of protein glycation by kavalactones and aminoguanidine is shown in Table 1. Both kawain (**1**) (IC_{50} = 43.5 ± 1.2 μM) and methysticine (**2**) (IC_{50} = 45 ± 1.3 μM) inhibited AGEs formation significantly better than aminoguanidine (**4**) (IC_{50} = 231.0 ± 11.5 μM; p = 0.01). Dehydromethysticine only showed poor inhibitory activity with (IC_{50} >500 μM). In order to ascertain if the test compounds prevented the formation of di-carbonyl moieties (glyoxal or methylglyoxal), the intermediate products of the Maillard reactions, test compounds were incubated with BSA and glucose for the indicated time. The formation of dicarbonyl adducts with Girard T was monitored spectrophotometrically. It was found that kawain (IC_{50} 356 ± 30 μM) and methysticin (IC_{50} 405 ± 44 μM) had a similar activity compared to aminoguanidine (IC_{50} 360 ± 50 μM; not significantly different, p = 0.01) (Table 1). The carbonyl compounds,

formed during the intermediate steps in the Maillard reaction, further interact with proteins to form the AGEs complex. Therefore, it was investigated if the test compounds could inhibit the interaction of glyoxal and protein (BSA). However, both kawain (IC_{50} 1.25 ± 0.03 mM) and methysticin (IC_{50} 1.36 ± 0.05 mM) had lower inhibitory activity than aminoguanidine (IC_{50} 0.25 ± 0.01 mM; $p = 0.01$) (Table 1).

Glucose-mediated protein glycation models are widely used to study AGEs inhibition. AGEs protein adducts can be formed under oxidative and non-oxidative conditions. Therefore, potential AGEs inhibitors are difficult to distinguish from general antioxidants such as many plant polyphenols [13]. Contrary to glycation of proteins by glucose, reactive carbonyl species (RCS) such as glyoxal and methyl glyoxal exhibit both extracellular and intracellular glycating properties, and are involved in non-oxidative glycation reactions and formation of AGEs *in vivo*. Therefore in the present study a glucose-based anti-glycation assay was used in parallel with a protein- glyoxal assay, which allows testing for pure glycation inhibitors, independent from antioxidative properties. Glycation inhibitors, the activity of which is based on antioxidative properties, may not effectively inhibit non-oxidative protein glycation, an important factor in intracellular and extracellular AGEs formation [14]. The *in vitro* IC_{50} values reported for aminoguanidine as an AGEs inhibitor usually are in the high-micromolar or even low-millimolar range, and much higher than the IC_{50} values of a plant polyphenol such as quercetine [15]. Nevertheless aminoguanidine has been the prototype for new drugs investigated both *in vitro* and *in vivo* to intervene with the formation of AGEs [16].

Glycation adducts of proteins are formed when proteins react with glucose-reactive α-oxoaldehydes or dicarbonyls, such as glyoxal, methyl glyoxal and 3-deoxyglucosone [17]. These adducts have recently been proposed to be formed from all stages of the glycation process, by degradation of glucose or Schiff's bases in early glycation, or from Amadori

products such as fructosamine in the intermediate stages of glycation. Therefore, inhibition of fructosamine and α-dicarbonyls formation, as well as preventing the further interaction of the dicarbonyls with protein could reduce AGEs formation. Out of several mechanisms of AGEs inhibition, entrapment of carbonyl moieties is well documented [18]. In order to determine whether the prevention of AGEs formation by kawain is due to the entrapment of dicarbonyl species, the loss of glyoxal in the course of the reaction was monitored. While aminoguanidine entrapped glyoxal, there was almost no entrapment of the carbonyl moiety by kawain (Fig. 2A). Aminoguanidine forms adducts with glyoxal and therefore, the relative amount of reactive glyoxal left decreased sharply within the first 2 h. However, even after incubating kawain with glyoxal for 6 h, there was no apparent reduction in the reactive glyoxal content, and therefore, it is evident that kawain does not entrap glyoxal.

Although kawain was found to have five times stronger inhibitory activity than aminoguanidine in preventing protein glycation, the kava lactone was less potent in preventing the interaction of glyoxal and BSA. This may be due to the incapability of kawain to entrap glyoxal. During the glyoxal-BSA interaction, aminoguanidine entraps glyoxal and therefore, the amount of reactive glyoxal remaining for the interaction with BSA to form AGEs is much less and hence the formation of glyoxal-BSA complex is reduced, which leads to higher inhibition. Similarly, the probable reason for the higher IC_{50} values of kawain obtained during the inhibition of carbonyl moiety generation is also related to carbonyl entrapment. Kawain could not entrap glyoxal and therefore, the amount of reactive glyoxal left for the formation of Girard adduct is higher, yielding greater IC_{50} values. It is evident from these results that the prevention of AGEs formation by kawain is not due to carbonyl entrapment.

Since entrapment of a carbonyl moiety was not found to be the mechanism of AGEs inhibition by kawain, and since DPPH free radical scavenging activity was also absent (results

not shown), the chelating activity of test compounds was investigated. It is reported that AGEs inhibitors primarily act as metal chelators that inhibit the metal-catalyzed oxidation reactions [19]. Therefore, firstly it was investigated if the test compounds prevent metal catalyzed ascorbate oxidation reactions. It was found that kawain (IC_{50} 57.2 ± 1.5 μM) prevented Cu^{2+} catalyzed ascorbic acid oxidation significantly better than aminoguanidine (IC_{50} 260.4 ± 5.3 μM) (Table 1; $p = 0.01$).

Furthermore, the kinetic studies of inhibition of ascorbic acid oxidation by kawain revealed a linear relationship in semilogarithmic plots (Fig. 2B). The result was similar to the prevention of ascorbate oxidation by aminoguanidine [20]. This indicated that both kawain and aminoguanidine have a similar mode of metal chelation towards Cu^{2+} ions in the ascorbic acid system.

After having established that kawain had metal chelating effects in the ascorbate oxidation system, it was investigated if the same effects could be studied in the glucose-BSA system. Therefore, first the fluorescence signals in the absence of inhibitor were monitored to understand if metal ions could increase AGEs formation in the glucose-BSA system. The addition of metal ions increased the fluorescence unit of the incubated samples in a dose-dependent manner (Fig. 3A). The fluorescence units were significantly higher in the presence of ions at concentrations of 50 μM or higher ($p = 0.01$). Previous studies have shown similar results and Cu^{2+} ions have been found to increase the AGEs formation in the glucose-collagen model [21]. Here, the effects of metal ions like $Fe^{3+}$and Cu^{2+} on AGEs formation using the glucose-BSA model are reported.

On the addition of inhibitors, the intensities of the fluorescence signals decreased sharply for both kawain and aminoguanidine. The IC_{50} values for AGEs inhibition by kawain and aminoguanidine were in the range of 10 – 25 μM and 75 – 140 μM respectively, for different ion concentrations (Fig. 3B). This implied that both compounds acted as chelators and

181 therefore inhibited the formation of AGEs. These data indicated that kawain acted as a better
182 chelator of Fe^{3+} and Cu^{2+} than aminoguanidine (Fig. 3B). The IC_{50} values decreased for both
183 test compounds on addition of metal ions, further supporting the fact that both compounds
184 prevent AGEs formation by metal chelation.

185 In order to further determine the degree of chelation by kawain and aminoguanidine, the
186 rate of metal chelation in the glucose-BSA model at different compound concentrations was
187 investigated. Previous studies on chelating kinetics using the ascorbate oxidation model have
188 been reported [20]; however, this is the first investigation of chelating kinetics in the glucose-
189 BSA model. The graphical analysis of the normalized chelation rate *versus* inhibitor
190 concentration showed that the percentage inhibition was a linear function of the square root of
191 the concentration for both Fe-catalyzed (Fig. 4A) and Cu-catalyzed (Fig. 4B) reactions. These
192 data yielded IC_{50} values of 114.3 and 328.2 μM for Fe-catalyzed and, 54.1 and 114.4 μM for
193 Cu-catalyzed AGEs formation inhibition by kawain and aminoguanidine, respectively.
194 Kawain had almost three times more Fe^{3+}- and two times more Cu^{2+}-ion chelating activity
195 than aminoguanidine. It must also be noted that the IC_{50} values obtained in Fe-catalyzed
196 reactions were much higher than in Cu-catalyzed AGEs formation. These values may suggest
197 that the test compounds have higher affinity towards the divalent Cu-ions than towards the
198 trivalent Fe-ions. It must be noted that the IC_{50} values obtained during chelation studies differ
199 from those obtained during kinetic studies. The former IC_{50} value is the concentration
200 required to prevent the 50% inhibition at a particular ion concentration, while the latter
201 described the degree of chelation.

202 Because kawain showed promising metal chelation activity, its effectiveness as an
203 inhibitor of Cu^{2+}-catalyzed oxidation reactions was also evaluated. Both kawain and
204 aminoguanidine had lower concentrations (2.5 – 10.0 μM) than in the AGEs experiments.
205 LDL oxidation was monitored by measuring malondialdehyde (MDA) equivalents generated

after incubation of LDL in a buffer containing Cu^{2+} ion and test samples. Both kawain and aminoguanidine prevented LDL oxidation significantly at concentrations of 5 μM or higher (p = 0.01) (Fig. 5A). The results also indicated that both kawain and aminoguanidine were equally effective in preventing Cu^{2+}-catalyzed LDL oxidation.

To investigate if kawain could also prevent lipid peroxidation without the addition of transition metals, the test compounds were incubated with linoleate in phosphate buffer at physiological pH, relying on endogenous metal ions in the buffer to catalyze linoleate oxidation. The oxidation was monitored by measuring the formation of TBARs and it was found that there was a substantial rise in TBARs values from day 1 to day 8, whereas, the treated samples showed an insignificant increase at the same time interval (Fig. 5B) (p = 0.01). These data also indicated that both kawain and aminoguandine have a similar efficacy in preventing linoleate oxidation. The prevention of lipid peroxidation in both the LDL and the linoleate system indicated that kawain not only prevented AGEs formation but also inhibited lipid peroxidation and thereby ALEs formation. This unique dual inhibitory property was reported for other compounds like pyridoxamine [22] and OPB–9195 [23].

In order to understand the efficacy of the test compounds in whole organisms, glucose toxicity was investigated in the nematode *Caenorhabditis elegans*. The worm is considered as a suitable model organism to study glucose toxicity, in which glucose condition limit the lifespan by increasing ROS formation and AGEs modification [24]. The worms were grown on high glucose plates and test samples of kawain (40μM) or aminoguanidine (250 μM). These concentrations lie in the range of the IC_{50} values for the respective compounds obtained during the *in vitro* AGEs inhibition. At these concentrations, the mean life span increased from 14.01 ± 1.48 days (untreated worms) to 16.77 ± 1.32 and 16.12 ± 1.34 days, respectively (Fig. 6A, Table 2). However, this lifespan extension was not statistically significant (p >

231 0.05). Therefore the concentrations of the test compounds were increased by two-fold and it
232 was found that the mean lifespan increased significantly to 17.84 ± 1.32 days (increase of
233 27.4%) for kawain, and to 17.53 ± 1.36 (increase of 25.2%) for aminoguanidine compared to
234 the control (Fig. 6B, Table 2, $p < 0.05$). These data indicate that kawain can increase the
235 lifespan of *C. elegans* under high glucose concentration possibly by modifying the AGEs
236 formation.
237
238 In conclusion, this study showed that kavalactones displayed significantly higher *in vitro*
239 AGEs inhibitory activity than a potent AGEs inhibitor, aminoguanidine. Prevention of AGEs
240 formation in different stages of the pathway was examined, and it was observed that
241 kavalactones have pronounced effects in all stages involving metal-catalyzed reactions (Fig.
242 7). Also the chelating kinetics of kawain and aminoguanidine were studied for the first time in
243 the glucose-BSA model. Since kawain neither exhibited carbonyl entrapment nor DPPH
244 radical scavenging activity, the inhibition of AGEs formation by kawain is predominantly due
245 to metal chelation. Furthermore, the prevention of lipid peroxidation was investigated and it
246 was established that the activity of kawain was similar to the activity of aminoguanidine in
247 preventing the formation of TBARs. Certain compounds like pyridoxamine, aminoguanidine
248 and OPB-9195 with dual inhibitory properties have always generated great interest in
249 researchers. Carnosine, a naturally occurring dipeptide with anti-glycation and metal
250 sequestering properties has been proposed as a possible candidate for drug design [25]. In this
251 regard, with anti-glycating, lipid peroxidation inhibiting, and metal chelating properties,
252 kavalactones can be considered as possible candidates for further research. The lifespan
253 extending properties of kawain under high glucose stress conditions in *C. elegans* certainly
254 confirm the importance of this group of compounds.
255

Materials and methods

All chemicals and test compounds, including kawain (>95% purity) and aminoguanidine (>98% purity) were purchased from Sigma-Aldrich unless stated otherwise. Methysticin (95.2% purity) and dihydromethysticin (96.4% purity) were kindly provided by Dr. Willmar Schwabe GmbH & Co.

Anti-glycation assay

The inhibition of protein glycation was measured as described previously [10], with slight modifications. The reaction mixture (500 μL) contained 400 μg BSA, 200 mM glucose and test compounds, in DMSO, at different final concentrations (10 – 500 μM). The mixtures were incubated at 37^{o}C for one week. Sodium azide (0.02%) was added to prevent bacterial growth. The change in fluorescence intensity (excitation 360 nm, and emission 450 nm) based on AGEs formation was monitored using a spectrofluorometer (Tecan Infinite M200). In order to reduce the interference in the fluorescence signal by the test compounds, parallel incubation at 4^{o}C was performed for all the samples. The AGEs inhibition was calculated as

$$\% \text{ AGEs inhibition} = [1-(S-Sb)/(C-Cb)] \times 100$$

where S and C represent relative fluorescence units (RFU) for test samples (in DMSO) and control (test mixtures containing only DMSO) incubated at 37^{o}C, and where Sb and Cb are RFU for samples incubated at 4^{o}C. The concentration required for 50% inhibition (IC_{50}) was determined graphically.

Protein-glyoxal interaction

Glyoxal (200 μL, 20 mM) was incubated with BSA (200 μL, 10 mg/mL) in the presence or absence of test compounds (200 μL) at 37 ^{o}C for one week. The inhibition of AGEs formation was determined as described above.

Determination of reactive carbonyl groups

The concentration of reactive carbonyl groups was determined using Girard-T reagent, with minor modifications [10]. Briefly, 100 μL aliquots of glycated materials were incubated at room temperature for 1 h with a reaction mixture containing 50 μL Girard-T solution (500 mM) and 850 μL of sodium formate (500 mM, pH 2.9). Fifty microliter of incubated sample was further diluted to 1 mL in the sodium formate buffer and the absorbance was measured at 290 nm using a spectrophotometer (Genesys 10 UV-Vis). Samples without Girard-T were used as blank. The inhibition was calculated as:

$[1 – (A_t – A_{tb}) / (A_c – A_{cb})] \times 100$,

where A_c and A_t are the absorbance of control and test samples, and A_{cb} and A_{tb} are their respective blanks.

Entrapment of reactive carbonyl compounds

The entrapment of glyoxal by test compounds was determined as described previously, with slight modifications [18]. Briefly, 10 mM glyoxal was incubated with 10 mM test compound at 37 ^{o}C in 200 mM sodium phosphate buffer, pH 7.4, containing 0.02% sodium azide. The loss of carbonyl moieties in the course of the reaction was measured spectrophotometrically using Girard-T reagent. Parallel experiments with carbonyl compounds incubated under the same conditions but without test compounds were used as references for calculating the relative amount of reactive glyoxal.

Ascorbic acid oxidation

To study if the test compound prevents metal-catalyzed ascorbic acid oxidation, kawain at various concentrations was incubated in a reaction mixture (1.5 mL) containing 500 μM ascorbic acid, 500 nM $CuCl_2$ in chelex-treated 50 mM phosphate buffer [19], pH 7.4, at room

306 temperature; reagents were pre-equilibrated at this temperature for 10 min. The loss of
307 ascorbic acid was monitored spectrophotometrically at 265 nm as described previously [26].
308
309 **Metal chelating activity**
310 The chelation activity of kawain and aminoguanidine was investigated in the glucose-
311 BSA system in catalytic metal-free buffer. Different concentrations of metal ions were added
312 in the presence and absence of test compounds. The samples were incubated at 37^{o}C for seven
313 days, with parallel incubations at 4^{o}C and the AGEs formation was monitored as described
314 above. For the kinetic studies, we used a time-driven protocol with initial velocity recorded
315 over a range of inhibitor concentrations.
316
317 **Metal catalyzed oxidation of LDL**
318 The metal catalyzed oxidation of LDL was investigated in microtiter plates as described
319 previously, with slight modifications [27]. Briefly, 100 μL of LDL (0.2 mg/mL in phosphate
320 buffered saline) was incubated at 37^{o}C with 10 μL of 55 μM $CuSO_4$ and 10 μL of test
321 compounds (0 – 1 mM) for 24 h. The reaction was terminated by addition of 10 μL of 1 mM
322 BHT and the samples were kept at –20^{o}C. The generation of malondialdehyde (MDA)
323 equivalents during LDL oxidation was estimated by the TBARs assay as described previously
324 [27].
325
326 **Oxidation of Linoleic acid (LA)**
327 LA (5 mM) was oxidized alone or in presence of kawain or amnoguanidine (1 mM) in 5
328 mL of 200 mM sodium phosphate buffer, pH 7.4 at 37^{o}C [22]. The reactions were stopped at
329 different time intervals (0, 1, 4, and 8 days), and the samples were analyzed immediately.

Aliquots were removed for the measurement of TBARS as described before [27], using MDA as an external standard.

Caenorhabditis elegans assay

The wild type *Caenorhabditis elegans* strain N2 (var. Bristol) and the *Escherichia coli* OP50 bacteria were obtained from *Caenorhabditis* Genetic Centre (University of Minnesota). The worms were routinely propagated at 20^{o}C on nematode growth medium (NGM) plates with *E. coli* strain OP50 as a food source. Synchronization of worm culture was achieved by treating gravid hermaphrodites with bleach (50% sodium hypochlorite; 2.5 M sodium hydroxide) and recovering the hatched L1 larvae on NGM/OP50 plates [28]. To prepare high glucose plates, a concentration of 40 mM glucose was added to the agar [24]. Synchronized worms were added to the NGM plates containing 5-fluorodeoxyuridine (to prevent progeny development) and test compounds at indicated concentrations. After 4 days, worms were transferred to new NGM/glucose plates and fed with live *E. coli* (OP-50) every two days to avoid starvation. Scoring was done every 2 days for the first 12 days, and every day thereafter. Survival was scored as the number of worms responsive to gentle touch as a fraction of the original number of animals on the plate.

Statistical treatment

All experiments were conducted in triplicate and the data represent the mean ± standard deviation. The IC_{50} value was determined graphically as the concentration of each sample required to give a 50% inhibitory activity. For significance analysis, the data were analyzed by one-way ANOVA and the means were separated using Tukey's HSD range test at p = 0.01. All statistical analyses were performed using SPSS version 20.0 for Windows.

Acknowledgments

The Special Fund for Research of the University of Antwerp, the National Fund for Scientific Research (FWO Flanders – Belgium) and the Flemish Agency for Innovation by Science and Technology (IWT) are kindly acknowledged for providing fellowships to Atul Upadhyay and Emmy Tuenter, respectively.

The authors declare they have no conflicts of interest.

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446 **Table 1**. IC_{50} values (in μM) of kavalactones and aminoguanidine preventing advanced and
447 intermediate glycated products and ascorbate oxidation.

	Kawain (**1**)	Methysticin (**2**)	Dehydromethysticin (**3**)	Aminoguanidine (**4**)
Protein glycation	43.5 ± 1.2 *	45.0 ± 1.3 *	> 500	231.0 ± 11.5
Dicarbonyl generation	356 ± 30	405 ± 44	nt	360 ± 50
GO-BSA interaction	1250 ± 30	1360 ± 50	nt	250 ± 1
Ascorbate oxidation	57.2 ± 1.5 *	nt	nt	260 ± 5

448 * indicates significant difference compared to the positive control aminoguanidine (Tukey test, p =
449 0.01); nt, not tested

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452

453 **Table 2**. Effects of kawain (KW) (**1**) and aminoguanidine (AG) (**4**) on the lifespan of *C.*
454 *elegans* under high glucose concentration. Values represent lifespan in days; SEM = standard
455 error of the mean.

Treatment	Conc (μM)	Total (N2)	Mean Estimate	Mean SEM	% Extended (compared to control)	Log-Rank χ^2	Log-Rank p
Control		121	14.01	1.48	-	-	-
KW (**1**)	40	115	16.77	1.32	19.79	2.28	0.13
	80	138	17.84	1.35	27.43	4.17	0.04
AG (**4**)	250	93	16.12	1.34	15.14	1.37	0.24
	500	126	17.53	1.36	25.21	4.01	0.04

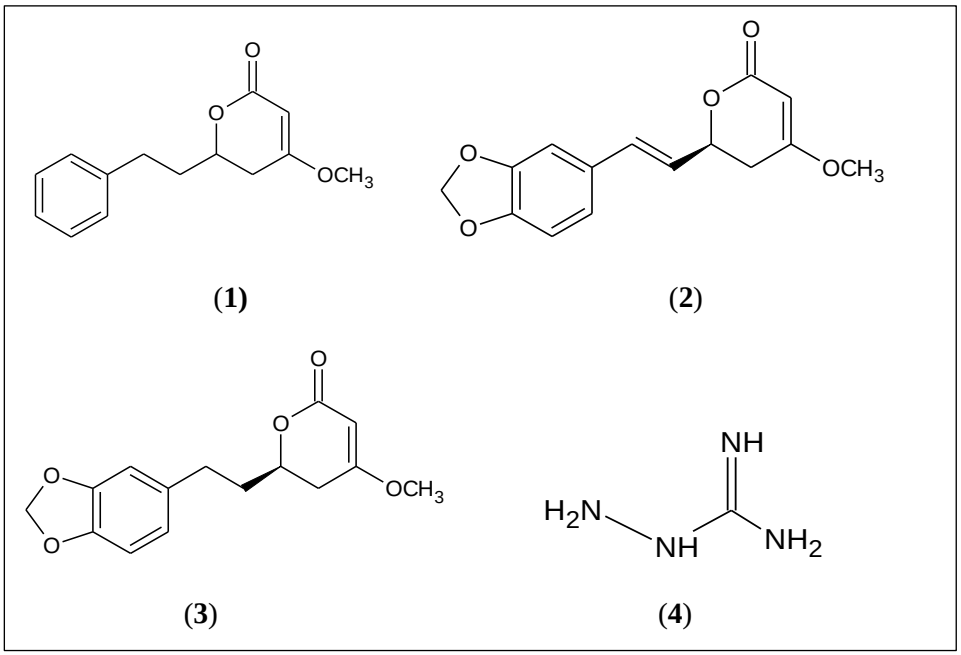
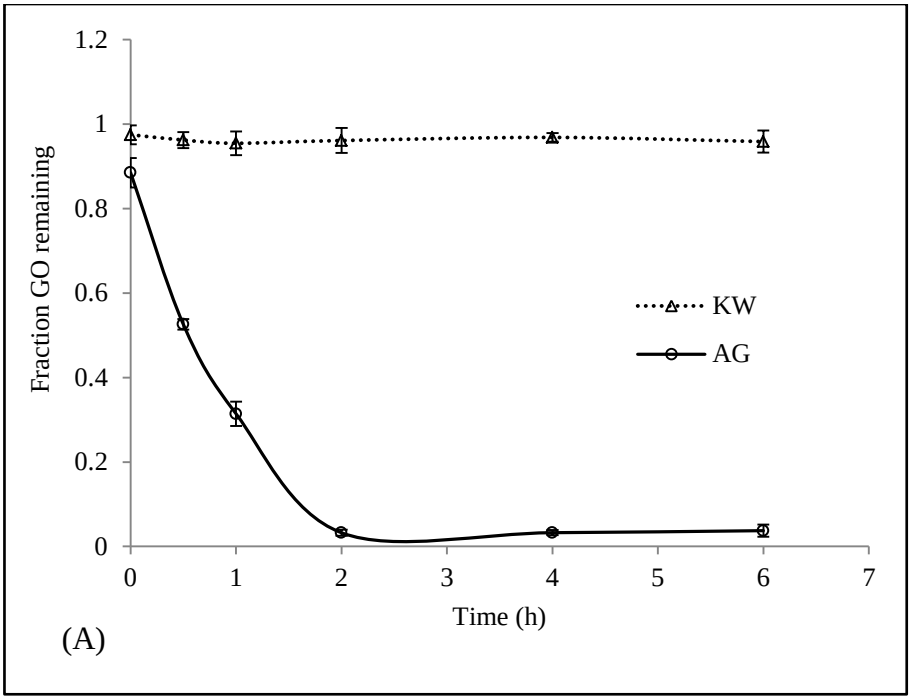


Fig. 1. Chemical structure of test compounds: kavalactones (DL-kawain (**1**); methysticin (**2**); dehydromethysticin (**3**); and aminoguanidine (**4**).



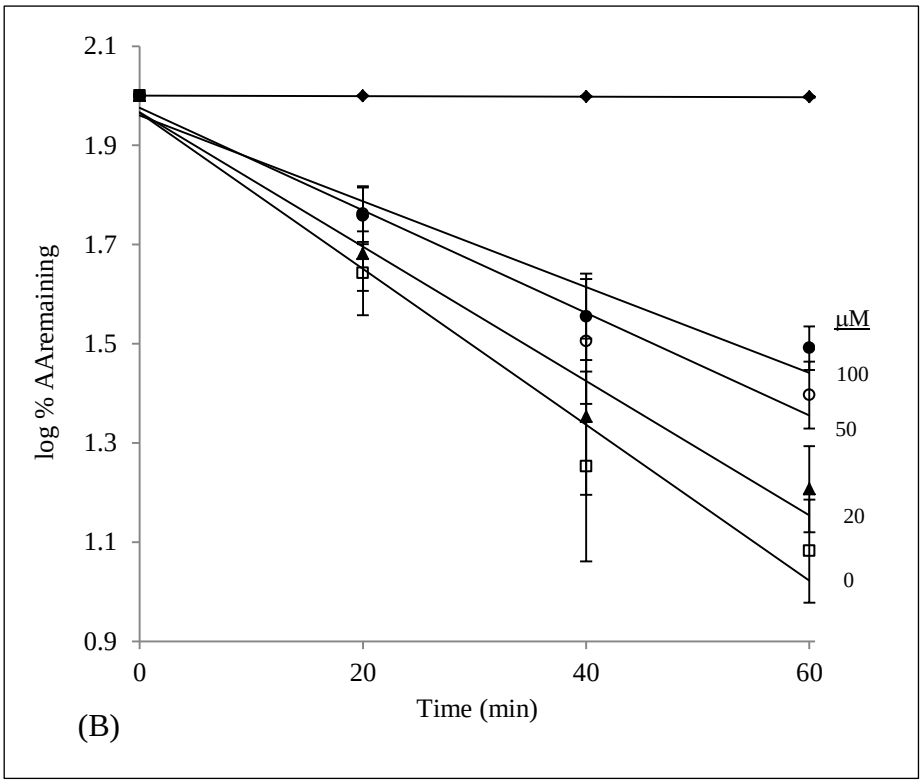
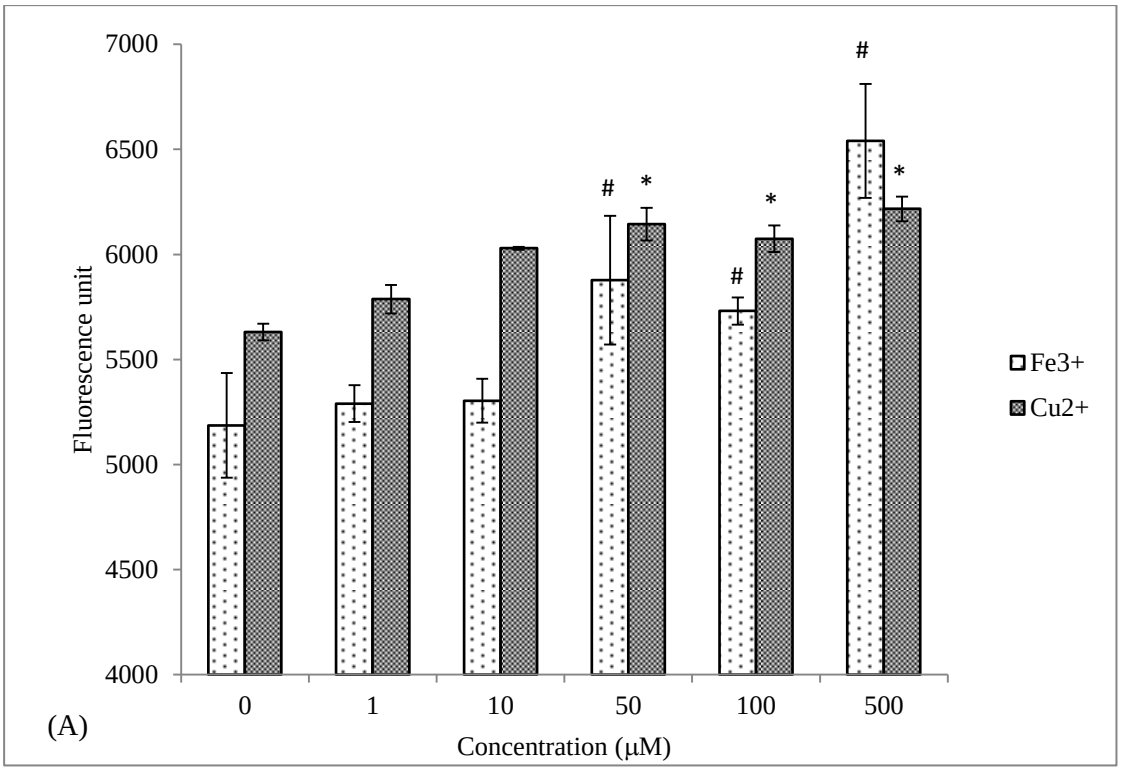


Fig. 2. Entrapment of glyoxal (GO) by aminoguanidine (AG) but not by kawain (KW) (A). Kinetics of oxidation of ascorbate (AA) in the absence and presence of various concentrations of kawain (B). Data points represent means ± SD of the triplicate experiments.



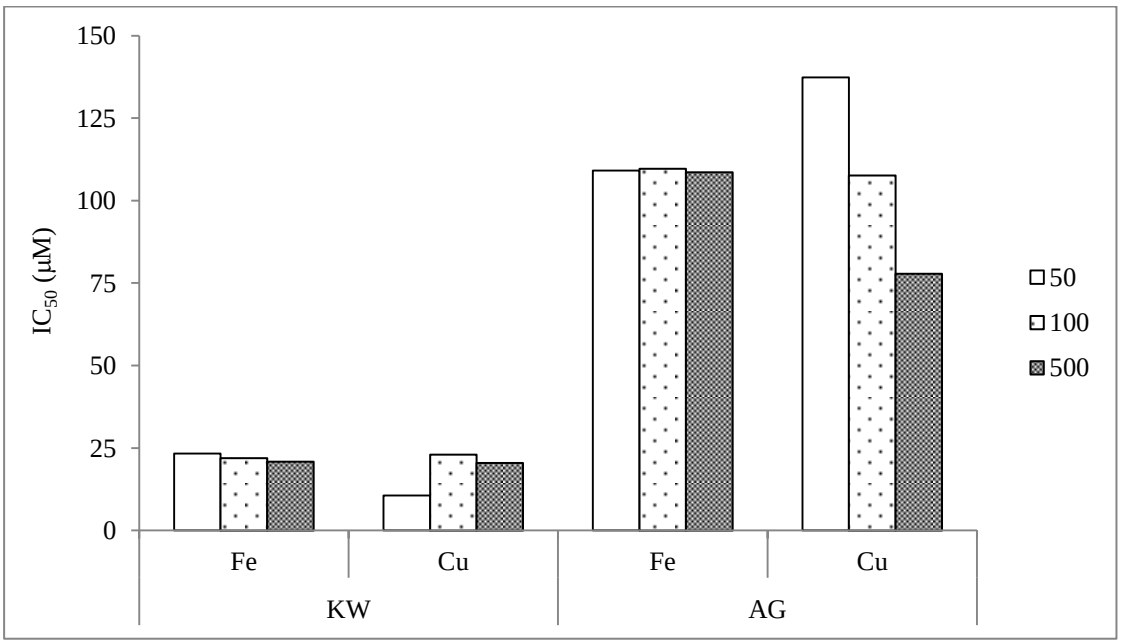
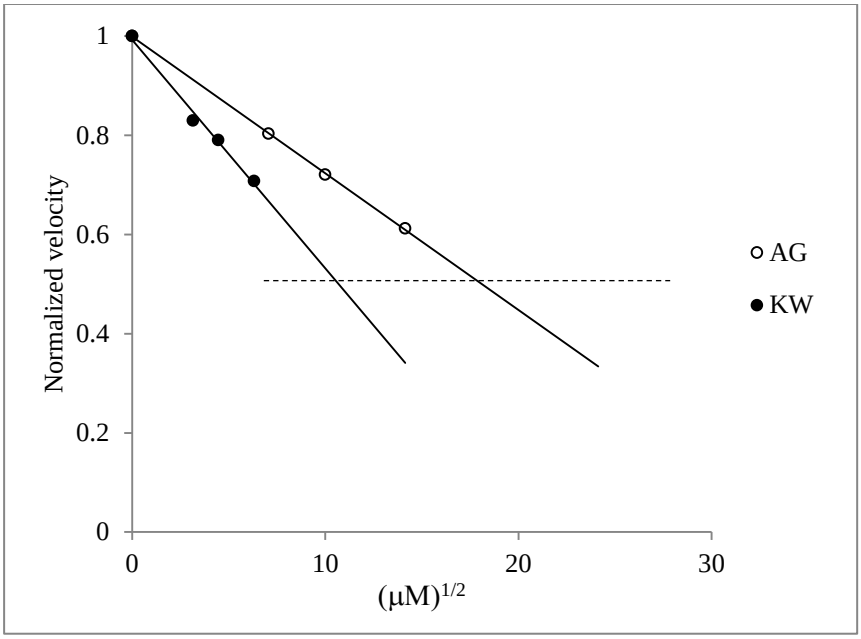


Fig. 3. Effect of metal ions in AGEs formation in the glucose-BSA system (A). Values represent mean ± SD of 3 data set; results with significant difference are indicated by # and * for Fe^{3+} and Cu^{2+} ions, respectively (p = 0.01). IC_{50} values of AGEs inhibition by kawain (KW) and aminoguanidine (AG) at different metal ion concentration (50 – 500 μM) (B).



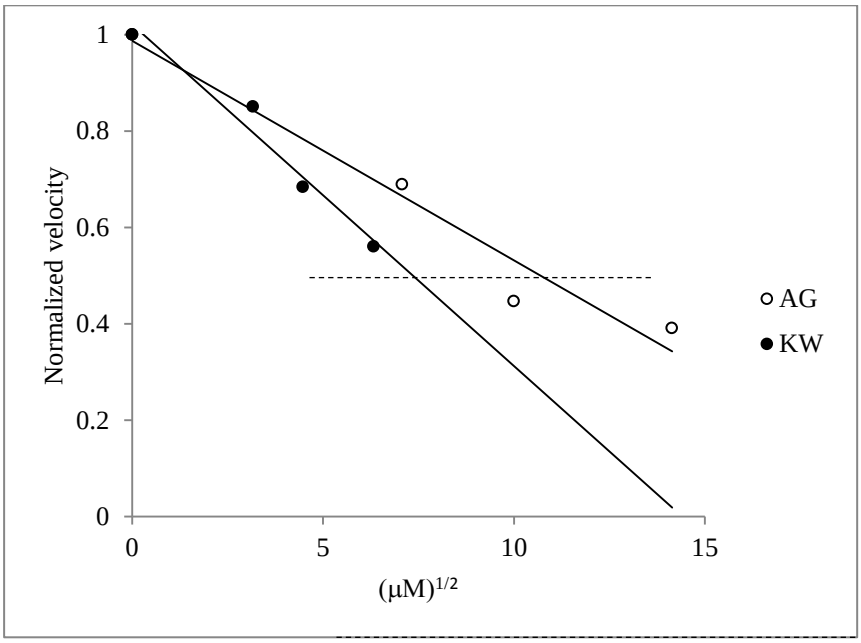
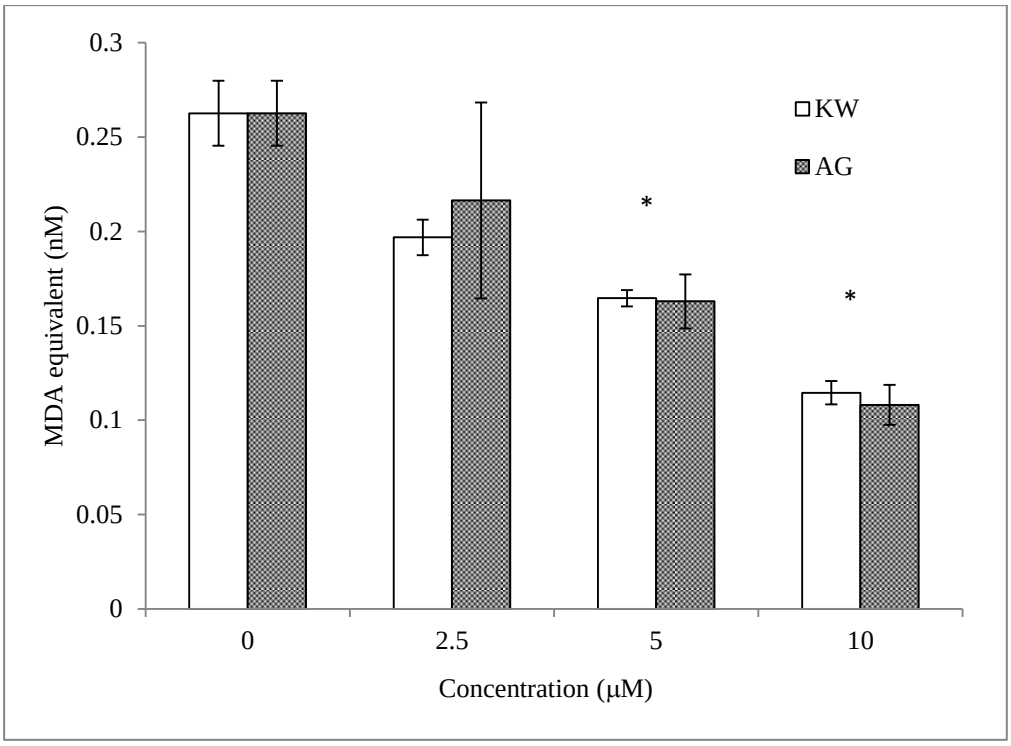


Fig. 4. Effect of kawain (KW) and aminoguanidine (AG) concentration on the kinetics of Fe-catalysed (A) and Cu-catalysed (B) AGEs formation.



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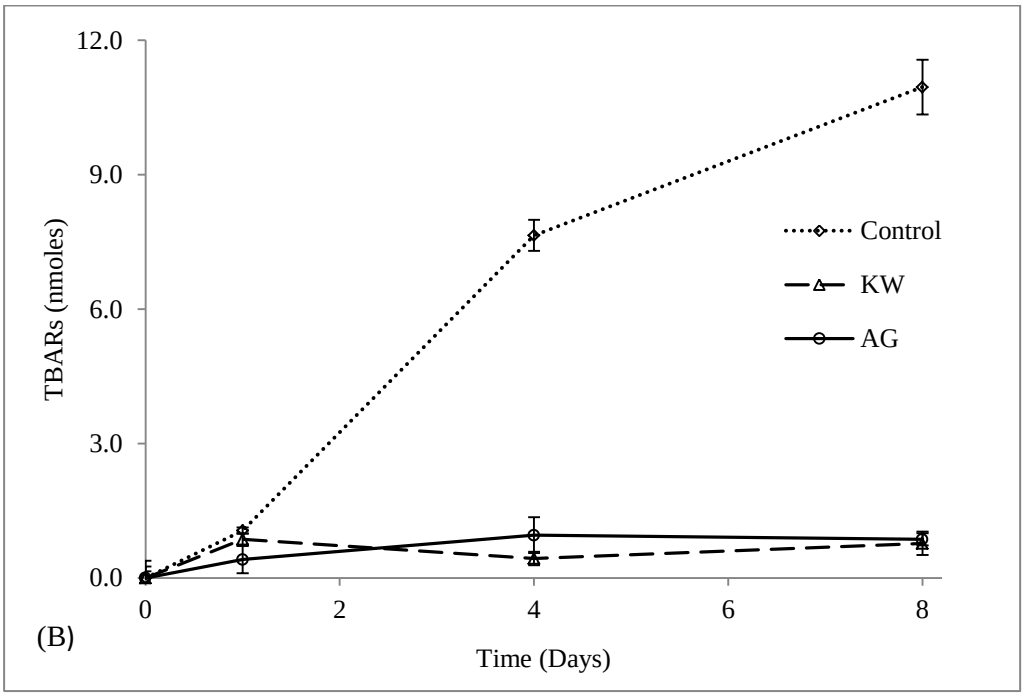

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502

503 **Fig. 5.** Inhibition of ALE formation by kawain (KW) and aminoguanidine (AG). Prevention

504 of LDL oxidation (A) and linoleate oxidation (B). The data represent mean ± SD of three

505 independent experiments. * Significant difference ($p < 0.01$).

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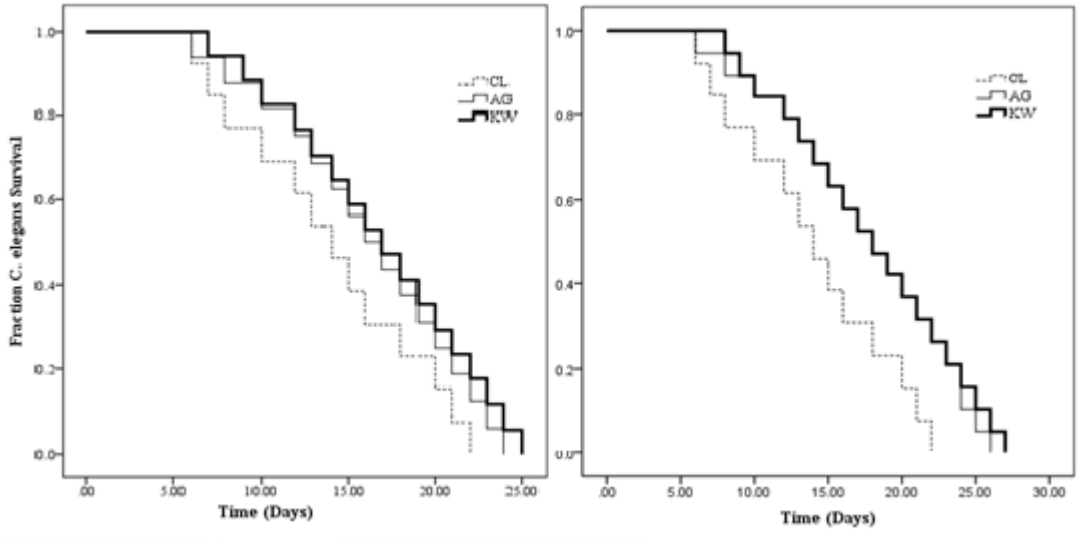

507

508 **Fig. 6**. Effect of kawain (KW) and aminoguanidine (AG) on the lifespan of *C. elegans*
509 exposed to a high glucose concentration. Kaplan-Meier graphs of the cumulative survival of
510 *C. elegans*. (A) represents lifespan, in days, of the worms pre-treated with KW and AG at
511 their IC_{50} concentrations, and (B) at higher concentrations. CL (control) represents non-
512 treated worms.

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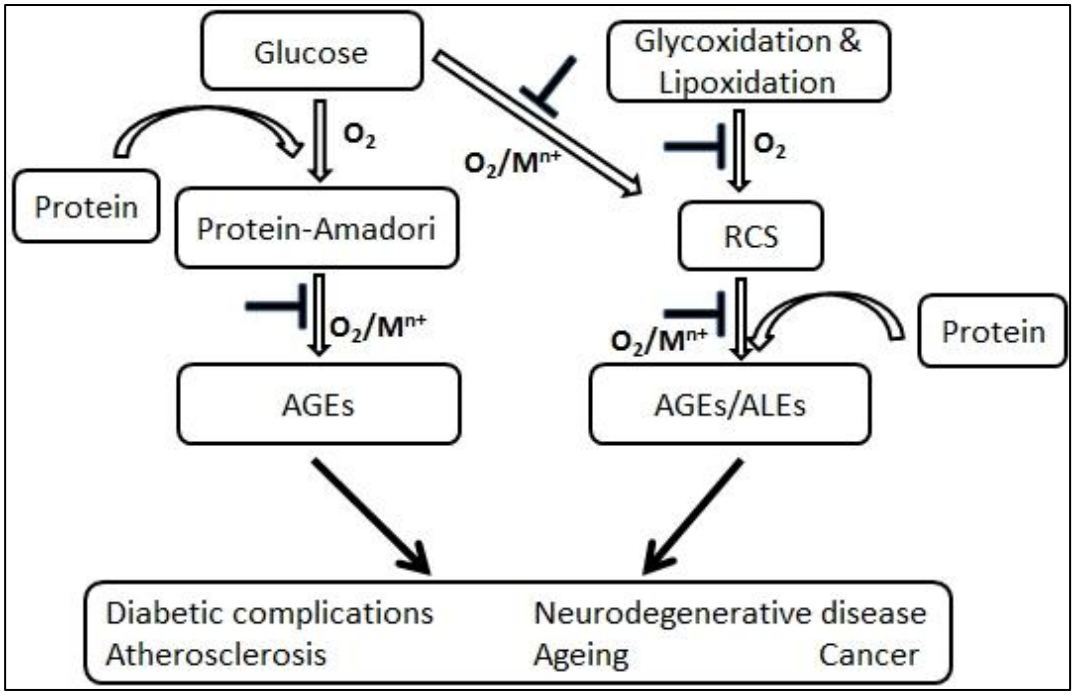

Fig. 7. Inhibition of AGEs or ALEs formation by kavalactones at different stages of the pathway.