Antibacterial and antimutagenic activities of novel zerumbone analogues

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Abstract

Zerumbone, the key constituent of Zingiber zerumbet Smith, is a very important bioactive phytochemical. Two new compounds viz. azazerumbone 1 and azazerumbone 2 were synthesised by ZnCl2-catalysed Beckmann rearrangement of the zerumbone oxime. The structure elucidation of these analogues of zerumbone was carried out by 1D (1H NMR and 13C NMR) and 2D-NMR (COSY, HSQC and NOESY) spectral analysis. Studies on the antibacterial activity established that azazerumbone 2 had better activity than zerumbone. Among the tested bacteria, Bacillus cereus was the most sensitive and Yersinia enterocolitica was found to be the most resistant. These compounds exhibited strong protection against sodium azide induced mutagenicity of Salmonella typhimurium strains TA 98 and TA 1531. Azazerumbone 2 showed better antibacterial and antimutagenic activity than azazerumbone 1. The antibacterial and antimutagenic activities exhibited by zerumbone and its analogues demonstrate their potential for use as nutraceuticals and in food preservation.

1. Introduction

Zingiber zerumbet Smith, belonging to the family Zingiberaceae, is a wild ginger variety, which is also known as medicinal ginger (Abdul et al., 2008). Though the rhizomes of Z. zerumbet are used as a flavouring agent in several parts of Asia, its medicinal properties have gained more attention recently. Traditionally, it is used to treat ailments like stomach-ache, toothache, swellings, strains, bruises and cuts. The key compound, zerumbone (Fig. 1, I), present in the Z. zerumbet is responsible for its bioactivity. Zerumbone has been tested for its bioactive attributes such as anticancer, anti-inflammatory and anti-HIV activities (Murakami, Takahashi, Ijiwajinda, Koshimizu, & Ohigashi, 1999; Tha et al., 2010). Hence, it has been a molecule of interest for chemists and biochemists in recent times. At present, studies are mainly focussed on exploring chemistry and understanding of the bioactivities of zerumbone and its analogues (Eltayeb et al., 2011; Songsiang, Pitchuanhom, Boonyarat, Hahnvajanawong, & Yenjai, 2010).

As zerumbone is the substance of natural origin having important bioactivity, chemical development is critical to comprehend diversity oriented synthesis (DOS). The DOS concept was introduced by Schreiber to define efficient synthesis of a library of small molecules capable of perturbing any disease related biological pathways (Schreiber, 2000). Zerumbone is a monocyclic sesquiterpene with three double bonds. Two of these are conjugated to the carbonyl group while one is an isolated one. The presence of α,β-unsaturated moiety in the zerumbone is considered to be a very important structural feature crucial for its anticancer properties (Murakami et al., 2002). Zerumbone is reported to exhibit potential anti-HIV and cytotoxic properties (EC50 ~0.04 μg/mL) and direct cytotoxicity to the target cells with IC50 ~0.14 μg/mL (Dai, Cardellina, McMahan, & Boyd, 1997). It has been a molecule of central focus because of its activity to inhibit growth of human leukaemia cell lines (Huang, Chien, & Wang, 2005) besides its antiproliferative activity (Alwi, Sakinah, Nallapan, Pihie, & Hawariah, 2007) activity. Few important derivatives of zerumbone have been synthesized to evaluate cytotoxicity against cholangiocarcinoma cell line. The derivatives with hydroxy amine, epoxy amine and nitrile functionalities exhibit very good activity whereas amino analogue exhibits an IC50 value of 16.44 μM against KKU-100 cell lines (Songsiang et al., 2010). Similarly, several synthetic transformations of this novel 11-membered ring system are reported (Kitayama, Masuda, et al., 2001; Kitayama, Yamamoto, et al., 2001). Tryptophan conjugate of the ring cleavage product of zerumbone exhibits inhibitory activity against Methicillin-resistant Staphylococcus aureus and Vancomycin-resistant Enterococci bacteria (Kitayama et al., 2007). The
chemical potential of zerumbone and its therapeutic applications has recently been reviewed (Kitayama, 2011; Prasannan et al., 2012).

In the present study, we envisaged the synthesis of new derivatives of zerumbone by utilising the reactive carbonyl functionality and its oxime. The Beckmann rearrangement of oximes, catalysed by Lewis acids/bases, is a well-studied chemical transformation as it offers a facile access to the incorporation of nitrogen into cyclic structures (Gawley, 1998). Also, zerumbol was obtained by the reduction of the carbonyl group of zerumbone (Kitayama, Masuda, et al., 2001; Kitayama, Yamamoto, et al., 2001). The antibacterial activities of these compounds against four food-borne pathogenic bacteria were evaluated and compared with zerumbone. Also, the anti-mutagenic activity of zerumbone and its analogues was evaluated against Salmonella tester strains.

2. Materials and methods

2.1. Materials and equipment

All the solvents and reagents used for the synthesis were of analytical grade. Fresh rhizomes of Zingiber zerumbet were collected from Indian Institute of Spices Research, Calicut, India. Zinc chloride, sodium borohydride and hydroxylamine hydrochloride were procured from Sigma Chemical Co. (St. Louis, MO, USA). $^1$H and $^{13}$C NMR spectra for the compounds were recorded on a 500 MHz NMR spectrometer (Bruker Avance, Reinstetten, Germany) using CDCl$_3$ solvent. The chemical shift values and coupling constants are given in $\delta$ and Hz respectively. Mass spectral analyses of the compounds were carried out using HRMS (Waters Q-Tof Ultima, Manchester, UK) in the ESI positive mode. The IR spectra (KBr pellets) were recorded on a FT Raman-Nicolet 5700 instrument. GC analyses were carried out on a Fisons 8000 instrument using a capillary column (OV-1, 30 m length; 0.25 mm i.d.; 0.25 $\mu$m film thickness) with injector and detector temperatures of 250 and $24.13\;\text{C}$ respectively. The oven temperature was held at 120 $\text{C}$ for 2 min and then raised at the rate of 3 $\text{C}$/min to 220 $\text{C}$ with a stay of 2 min at the final temperature. Thin-layer chromatographic (TLC) analyses were performed on silica gel 60 F$_{254}$ (Merck, Germany) coated on alumina sheet with 2–10% ethyl acetate in petroleum ether (60–80 $\text{C}$) as the developing solvent. The crude products were purified by column chromatography on silica gel (100–200 mesh) with ethyl acetate in petroleum ether (60–80 $\text{C}$) as eluting solvent. All the chemicals and petri-plates used for microbial studies were procured from Hi Media Ltd., Mumbai, India.

2.2. Isolation of zerumbone

Fresh rhizomes of Zingiber zerumbet (1 kg) were washed thoroughly, peeled and crushed to a paste. It was charged to a 3 L round bottom flask and subjected to hydro-distillation (4 h). The oil collected (10 mL) was separated and taken for purification by silica gel column chromatography (100–200 mesh). The pure product obtained from the column was dried in a desiccator over anhydrous CaCl$_2$ for 12 h. The colourless zerumbone crystals (4 g) had physical and spectral characteristics which are in accordance with the literature values (Dev, 1960).

2.3. Synthesis of zerumbone oxime

To a solution of zerumbone (0.3 g, 1.376 mmol) in ethanol (10 mL), hydroxylamine hydrochloride (0.96 g, 13.76 mmol) and K$_2$CO$_3$ (1.9 g, 13.76 mmol) were added at room temperature. The mixture was stirred and the progress of the reaction was monitored by TLC using 2% ethyl acetate in hexane. After completion of reaction (5 h), the reaction mixture was filtered and the residue was washed with ethanol. The filtrate was concentrated under reduced pressure to afford a syrupy mass, which was dissolved in dichloromethane (10 mL). The organic layer was washed with water (10 mL $\times 3$) and dried over anhydrous sodium sulphate. The resultant clear solution was concentrated to afford a material which was directly taken for next stage without further purification (0.25 g, 90%).

Zerumbone oxime (mixture of E- and Z-isomers): White powderr; m.p. 172–174 $\text{C}$; $^1$H-NMR (500 MHz, DMSO-d$_6$): $\delta$ = 10.56 (bs, 1H, N–OH), 6.14 (d, 1H, $J$ = 16.4 Hz, C11–H), 5.38 (d, 1H, $J$ = 16.6 Hz, C10–H), 5.28 (bs, 1H, C3–H), 5.15 (t, 1H, J = 7.2 Hz, C7–H), 2.08–2.32 (br, 6H, CH$_2$), 1.78 (s, 3H, C13–H$_3$), 1.46 (s, 3H, C12–H$_3$), 1.02–1.18 (br, 6H, CH$_2$). $^13$C-NMR (125 MHz, DMSO-d$_6$): $\delta$ = 160.23 (C1), 151.87 (C10), 138.70 (C3), 135.33 (C6), 133.44 (C2), 124.20 (C7), 120.83 (C11), 39.47 (C5 and C8), 36.26 (C9), 29.96 (C14), 24.13 (C15), 23.39 (C4), 15.19 (C12), 15.02 (C13); IR (v cm$^{-1}$): 3217 (br, –OH), 1636 (C=O). HRMS: [M$^+$+1] for C$_{15}$H$_{23}$NO, Calculated: 234.1780, found: 234.1761.

2.4. Synthesis of zerumbol

To zerumbone (0.3 g, 1.376 mmol), taken in methanol (10 mL) and cooled to 0 $\text{C}$, sodium borohydride (0.21 g, 5.52 mmol) was added slowly and the mixture was stirred at the same temperature. Progress of the reaction was monitored by TLC analyses using 2% ethyl acetate in hexane. After the completion of the reaction (1.5 h), mixture was concentrated. Ethyl acetate (10 mL) was added to the resulting syrupy liquid. The organic layer was separated and washed with water (10 mL $\times 3$). It was then dried over anhydrous sodium sulphate and concentrated to afford viscous oil. It was purified by column chromatography over silica gel (200–400 mesh) with mixture of ethyl acetate and petroleum ether to afford the pure product as a white crystalline solid. The physical and spectral characteristics of pure compound conformed well to the reported values (Kitayama, Masuda, et al., 2001; Kitayama, Yamamoto, et al., 2001).
2.6. Antimicrobial studies of zerumbone derivatives

The antibacterial assay of zerumbone and its derivatives was carried out by pour plate method against *Salmonella typhimurium* (TA 98 and TA1538) by standard plate incorporation test as described by Maron and Ames (1983). The antibacterial assay was based on the determination of number of His+ revertants in the plate. The test samples (3125, 625, 1250, 2500, 3750, 5000, 6250 and 7500 μg) were assayed by plating with molten soft agar (2 ml) containing 0.5 mM of histidine/biotin and 0.1 ml of 10 h old culture of strains of *S. typhimurium* onto minimal glucose agar plates. Sodium azide was used as a positive control (1.5 μg plate) in positive control and plates without test samples and sodium azide were considered as negative controls. Counts of His+ revertants were taken after incubation of the plates at 37 °C for 48 h. The mutagenicity of sodium azide in the absence of test samples was defined as 100% or 0% inhibition. The % inhibition was calculated using the formula % Inhibition = \( \frac{1 - T/M}{T} \times 100 \), where T is number of revertants per plate in presence of mutagen and test sample, and M is number of revertants per plate in positive control. The number of spontaneous revertants (counts in negative control) was subtracted from both numerator and denominator. The data of mean ± SD of three independent assays for each test sample are presented here. The antibacterial effect was taken as weak when the inhibitory effect was less than 25%; medium when the inhibitory effect was 25–40% and strong when the inhibitory effect was more than 40% (Ikken et al., 1999).

2.7. Antimutagenicity by Ames test

The antimutagenicity test for zerumbone and its analogues was carried out using the tester strains of *Salmonella typhimurium* (TA 98 and TA1538) by standard plate incorporation test as described by Maron and Ames (1983). The antimutagenicity assay was based on the inhibition of mutagenicity of sodium azide by the test samples as calculated by determining the number of His+ revertants in the plate. The test samples (3125, 625, 1250, 2500, 3750, 5000, 6250 and 7500 μg) were assayed by plating with molten soft agar (2 ml) containing 0.5 mM of histidine/biotin and 0.1 ml of 10 h old culture of strains of *S. typhimurium* onto minimal glucose agar plates. Sodium azide was used as a positive control (1.5 μg plate) in positive control and plates without test samples and sodium azide were considered as negative controls. Counts of His+ revertants were taken after incubation of the plates at 37 °C for 48 h. The mutagenicity of sodium azide in the absence of test samples was defined as 100% or 0% inhibition. The % inhibition was calculated using the formula % Inhibition = \( \frac{1 - T/M}{T} \times 100 \), where T is number of revertants per plate in presence of mutagen and test sample, and M is number of revertants per plate in positive control. The number of spontaneous revertants (counts in negative control) was subtracted from both numerator and denominator. The data of mean ± SD of three independent assays for each test sample are presented here. The antimutagenic effect was taken as weak when the inhibitory effect was less than 25%; medium when the inhibitory effect was 25–40% and strong when the inhibitory effect was more than 40% (Ikken et al., 1999).
resolution mass which was in close agreement with the calculated mass (M+1 for C15H23NO, calculated: 234.1780, found: 234.1813).

The minor product isolated by column chromatography had a doublet signal at 7.86 ppm (J = 9.8 Hz) in the HSQC spectrum with no attached carbon. This peak was assigned to the –NH– moiety of the molecule. The double doublet nature of the signal at 6.08 ppm indicates the olefinic proton at C-11 is coupled to both NH (J = 9.8 Hz) and vicinal proton at C-10 (4.79 ppm, J = 14.5 Hz). The 1H–1H COSY is presented in Fig 2c. This clearly demonstrates that –NH– insertion occurred in between C1 and C-11. Further, a strong NOE was observed between the amide proton and olefinic protons at C-10 and C-11 (Fig. 2d). The gem dimethyl at position C-9 exhibited strong NOE with C-7 and C-10 olefinic protons at 5.15 ppm and 4.79 ppm respectively. The NOE was also observed with C-8 methylene protons at 2.11 ppm. The IR analysis ascertained the presence of carbonyl (1666 cm−1) and –N–H (1640 cm−1) functionalities. All the structural features led to the elucidation of the structure of the compound and it was named as azazerumbone 1.

Further, it was confirmed by high resolution mass which is in close agreement with the calculated mass (M+1 for C15H23NO, calculated: 234.1780, found: 234.1751). The E- and Z-isomers elute collectively in the column chromatography and hence difficult to separate. The formation of two products in the reaction of zerumbone oxime under Beckmann rearrangement conditions is attributed to the specific orientation of hydroxyl group attached to nitrogen in E- and Z- isomeric forms. Beckman rearrangement is catalysed by an acid and in the present reaction, zinc chloride complexes with the hydroxyl group. The alkyl moiety of the ring anti to the OH group cleaves in a stereospecific fashion and migrates to nitrogen with concurrent ring expansion (Prakash, Mathew, & Olah, 2012). This is followed by the attack of negatively charged oxygen species on the carbenium ion linked to nitrogen resulting in the formation of an iminol which tautomerises to a carbonyl moiety and the lactam ring formation. Thus, the formation of azazerumbone 2 and azazerumbone 1 from E- and Z-oximes respectively can be explained as depicted in Fig 3.

This was further confirmed by GC analysis of the reaction product at periodic intervals. At the start of the reaction, E- and Z-oximes with retention times of 26.9 and 27.2 min were in relative ratios of 76% and 24% respectively. At 0.5 h, their relative ratios were 40% and 14% along with two products at retention times of 30.3 and 31.3 min in 31.4% and 20% ratio by composition (GC profiles provided in the supplementary information). The major product at this stage was purified and identified as azazerumbone 2, whereas the minor product was identified as azazerumbone 1. The progressive reduction of the two oxime isomers in the reaction along with a concomitant increase of the corresponding products further corroborated the formation of azazerumbone 2 from E-isomer and azazerumbone 1 from Z-isomer.

3.2. Antibacterial activity of zerumbone analogues

Antibacterial activity of zerumbone analogues was evaluated and compared with zerumbone. Zerumbone was most effective against Escherichia coli, but showed higher MIC values for other bacteria. Zerumbol was the most effective one among all the tested compounds as it showed minimal MIC values against all the tested bacteria (Table 1). However, when compared to zerumbone, zerumbol showed statistically similar growth inhibition against B. cereus and Y. enterocolitica and lower inhibition of E. coli and S. aureus growth at 25 ppm (Fig. 4). Azazerumbone 1 exhibited least antibacterial activity and could not inhibit the growth of the tested
bacteria effectively even at 1000 ppm. Azazerumbone 2 showed MIC similar to that of zerumbol against Gram-negative bacteria, but showed higher MIC values against Gram-positive bacteria. At lower concentrations too (25 and 50 ppm), both the compounds resulted in statistically similar growth inhibition pattern against Gram-negative bacteria, but higher inhibition of growth of Gram-positive bacteria was observed by zerumbol. Zerumbone oxime showed similar MIC value as zerumbol against B. cereus but had much higher MIC against other bacteria. At 50 ppm concentration, it showed statistically higher inhibition against B. cereus and S. aureus and lower inhibition of E. coli growth as compared to zerumbol.

The compounds tested in the present study showed variable activity against different bacteria. Among the tested bacteria, B. cereus was the most sensitive whereas Y. enterocolitica was most resistant to tested compounds. Zerumbone oxime showed similar MIC against E. coli and S. aureus and Azazerumbone 2 showed similar MIC against E. coli and B. cereus. E. coli was most resistant to zerumbone oxime, whereas S. aureus was more resistant to zerumbone. In general, Gram-positive bacteria are considered more sensitive than Gram-negative bacteria to different antimicrobial compounds because of the differences in the structure of their cell wall composition (Nikaido & Vaara, 1985; Scherrer & Gerhardt, 1971). But in the present study, no definite trend was observed for Gram-positive and Gram-negative bacteria. Although Y. enterocolitica, a Gram-negative bacterium, was most resistant to all compounds, another Gram-negative bacterium, E. coli, did not respond similarly and sometimes showed similar or lower activity as compared to S. aureus and B. cereus, the Gram-positive bacteria tested in present study. The similar observation of variable activity has been reported earlier for various plant extracts (Negi, 2012) and synthetic compounds (Parvathy, Negi, & Srinivas, 2010; Manjunatha et al., 2013; Parvathy et al., 2010). Various phenolic compounds such as ellagic acid, catechins and chlorogenic, caffeic and ferulic acids act as potent antimutagen and are reported to be inhibitors of chemical carcinogenesis (Ayrton, Lewis, Walker, & Loannides, 1992; Edenharder & Tang, 1997; Surh, 2002). In the present study, zerumbone and its derivatives showed strong antimutagenic activity (at and above 625 µg/plate level) in this study (Fig. 5a).

Zerumbone showed minimal antimutagenic effect against sodium azide induced mutation in S. typhimurium TA 1538 strain as it showed weak activity even at 6250 µg/plate level (Fig. 5b), and the activity was statistically lower than other compounds at all the concentrations tested. Zerumbol and azazerumbone 2 showed weak activity below 1250 µg/plate level; however zerumbol and azazerumbone 1 at and above 2500 µg/plate level, and azazerumbone 2 at and above 625 µg/plate level showed strong antimutagenic activity. All the compounds showed higher antimutagenic activity against S. typhimurium TA 98 as compared to S. typhimurium TA 1538. Similarly among various compounds tested, azazerumbone 1 and azazerumbone 2 showed best antimutagenic activity against S. typhimurium TA 98, azazerumbone 2 and zerumbone oxime were best against S. typhimurium TA 1538.

Antimutagenic activity of several natural as well as synthetic compounds is reported in literature (Negi, Jayaprakasha & Jena, 2003; Manjunatha et al., 2013; Parvathy et al., 2010). Various phenolic compounds such as ellagic acid, catechins and chlorogenic, caffeic and ferulic acids act as potent antimutagen and are reported to be inhibitors of chemical carcinogenesis (Ayrton, Lewis, Walker, & Loannides, 1992; Edenharder & Tang, 1997; Surh, 2002). In the present study, zerumbone and its derivatives showed strong antimutagenic activity (at and above 625 µg/plate concentration) indicating their anticancer potential as it is envisaged that compounds that possess antimutagenic activity can also inhibit cancer (Ikken et al., 1999).

3.3. Antimutagenic activity of zerumbone analogues

Zerumbone oxime showed weak antimutagenic effect against sodium azide induced mutation in S. typhimurium TA 98 strain even at 1250 µg/plate, however, at and above 3750 µg/plate level it showed strong antimutagenic activity, but the activity was statistically lower than other compounds at 156–5000 µg/plate level. At 6250 µg/plate level, all the compounds showed similar antimutagenic activity. Zerumbone and zerumbol had statistically similar antimutagenic activity at 156 and 312.5 µg/plate level, but zerumbol showed significantly higher activity at 625 and 1250 µg/plate level. At and above 2500 µg/plate level both the compounds showed similar activity. Azazerumbone 1 and azazerumbone 2 were best antimutagenic compounds as both showed strong antimutagenic activity, even at the lowest concentration tested (156 µg/plate level) in this study (Fig. 5a).

Zerumbone showed minimal antimutagenic effect against sodium azide induced mutation in S. typhimurium TA 1538 strain as it showed weak activity even at 6250 µg/plate level (Fig. 5b), and the activity was statistically lower than other compounds at all the concentrations tested. Zerumbol and azazerumbone 2 showed weak activity below 1250 µg/plate level; however zerumbol and azazerumbone 1 at and above 2500 µg/plate level, and azazerumbone 2 at and above 625 µg/plate level showed strong antimutagenic activity. All the compounds showed higher antimutagenic activity against S. typhimurium TA 98 as compared to S. typhimurium TA 1538. Similarly among various compounds tested, azazerumbone 1 and azazerumbone 2 showed best antimutagenic activity against S. typhimurium TA 98, azazerumbone 2 and zerumbone oxime were best against S. typhimurium TA 1538.

Antimutagenic activity of several natural as well as synthetic compounds is reported in literature (Negi, Jayaprakasha & Jena, 2003; Manjunatha et al., 2013; Parvathy et al., 2010). Various phenolic compounds such as ellagic acid, catechins and chlorogenic, caffeic and ferulic acids act as potent antimutagen and are reported to be inhibitors of chemical carcinogenesis (Ayrton, Lewis, Walker, & Loannides, 1992; Edenharder & Tang, 1997; Surh, 2002). In the present study, zerumbone and its derivatives showed strong antimutagenic activity (at and above 625 µg/plate concentration) indicating their anticancer potential as it is envisaged that compounds that possess antimutagenic activity can also inhibit cancer (Ikken et al., 1999).

Table 1

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<thead>
<tr>
<th>Compound</th>
<th>Concentration (ppm)</th>
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<td></td>
<td>B. cereus</td>
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<tr>
<td>Zerumbone</td>
<td>100</td>
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<tr>
<td>Zerumbol</td>
<td>60</td>
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<tr>
<td>Azazerumbone 2</td>
<td>75</td>
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<tr>
<td>Zerumbone oxime</td>
<td>60</td>
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* Result of four experiments (concentration at which no growth was observed).

b Azazerumbone 1 did not inhibit complete growth of any of the tested bacteria even at 1000 ppm concentration.
The variable activity of zerumbone and its analogues appears related to the molecular structure of the compounds. Zerumbone possesses good antibacterial activity but exhibits low antimutagenic effect against sodium azide induced mutation in *S. typhimurium* TA 1538 even at 6250 µg/plate level. Chemical modification of the reactive carbonyl moiety has resulted in the increased activity.

Fig. 4. Anti-bacterial activity of zerumbone and its analogues against *B. cereus*, *S. aureus*, *E. coli*, and *Y. enterocolitica* (values followed by same letters for each concentration are not significantly different (*p* < 0.05).

Fig. 5. Inhibitory effect of zerumbone and its analogues against sodium azide induced mutagenicity in *S. typhimurium* tester strains (a) TA 98 and (b) TA 1538 (values followed by same letters for each concentration are not significantly different (*p* < 0.05).
as exhibited by zerumbol. While the oxime exhibited activity comparable to zerumbone, the introduction of –NH— functionality in the ring has greatly influenced the activity of zerumbone. The position of this moiety has a major effect on the activity of zerumbone demonstrating an interesting structure–activity relationship.

In conclusion, a facile synthesis of two new analogues of zerumbone with amide moiety in the 12-member cyclic ring system has been developed. Azazerumbone 2, a major product of Beckmann rearrangement of E-zerumbone oxime, exhibited better antibacterial and antimitugenic activity than zerumbone. Zerumbone oxime and zerumbol showed comparable or better bioactive attributes than zerumbone. The introduction of –NH— group in the zerumbone ring influenced the activity of the molecule as evidenced by in vitro bioactivity assays. Its presence on either side adjacent to the reactive carbonyl moiety affected its bioactive properties in a specific fashion. The antimitugenic activity of zerumbone and its analogues demonstrate that they could be good candidates for nutraceutical applications and food preservation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.04.021.

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