African Journal of Biotechnology Vol. 9(8), pp. 1260-1265, 22 February, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Validated high performance liquid chromatographic (HPLC) method for analysis of zerumbone in plasma

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Accepted 11 November, 2009

Zerumbone (ZER) is a sesquiterpene derived from Zingiber zerumbet smith, family Zingiberaceae. It has been shown to possess anti-cancer and apoptosis-inducing properties against various human tumour cells as well as *in vivo* against a number of induced malignancies in mice. In this study a simple, specific and accurate high performance liquid chromatographic method for determination of ZER in micro-volumes human plasma (| 1.5 ml) was developed and validated. ZER and its analogue α-Humulene as internal standard were easy recovered by simple one step plasma protein precipitation using acetonitrile and separated in isocratic mobile phase, on reverse phase-C_{18} column. The effluent was monitored by Photodiode Array (PDA) detector and at a flow rate of 1.0 ml/min. The linearity of proposed method was 2 – 15 µg/ml. The intra-day and inter-day coefficient of variation and percent error values of the method were less than 15% and mean recovery was more than 90% for both ZER and α-Humulene. This method was found to be precise, specific, accurate and robust for detection and analysis of ZER in human plasma.

Key words: ZER, humulene, HPLC, human plasma.

INTRODUCTION

Zingiber zerumbet (L.) smith, known as lempoyang, is a member of the family Zingiberaceae, used in the traditional medicine as a cure for swelling, sores, loss of appetite and worm infestation in children (Somchit and Nur-Shakirah, 2003). In some Southeast Asian countries, the rhizomes of the plant are employed in traditional medicine as anti-inflammation, while the young shoots and inflorescence are used as condiments (Murakami et al., 2002). This plant has been shown to have anti-tumor (Sakinah et al., 2007), anti-inflammatory (Murakami et al., 2002) and cyclooxygenase-2 suppressant properties (Tanaka et al., 2001). Zerumbone (ZER) is a bioactive crystalline monocyclic sesquiterpene derived from zingiber zerumbet rhizomes. It exhibits variety of interesting reactions, such as region-and regeoselective conjugates additions, transannular ring contraction, cyclization and several regeospecific reactions which cleave the 11-membered ring (Kitayama et al., 1999, 2001; Ohe et al., 2000). This bioactive component has its unique structure, with cross-conjugated ketone in an 11-membered ring, as well as remarkable biological activities. It has been reported that ZER constitutes about 37% of *Z. zerumbet* (Matthes et al., 1980; Sakinah et al., 2007). Moreover, this compound showed a potential candidate for the development of anti-cancer treatment of natural origin.

ZER has been previously identified as distinct suppressor of tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced Epstein-Barr virus

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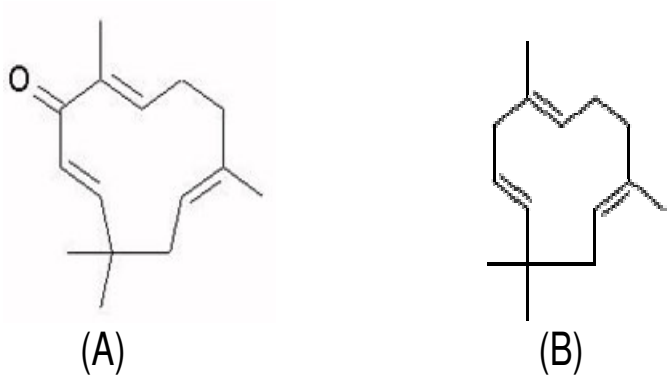

Figure 1. Chemical structure of Zerumbone (A) and α-Humulene (B).

(EBV) activation in Raji cells (Murakami et al., 1999). Murakami et al. (2004) reported that ZER inhibits the proliferation of colon and skin cancer cells through apoptosis, while having less effect on normal cells proliferation (Murakami et al., 2002; Murakami et al., 2004). Recently, ZER was reported to suppress the development of cervical intraepithelial neoplasia in mouse (Abdul et al., 2008). ZER has gained a great attention due to its activity towards many diseases *in vitro* and *in vivo*. Very recently, Sung et al. (2009) reported ZER as modulator for osteoclastogenesis induced by Receptor Activator for Nuclear Factor κ B Ligand (RANKL) and breast cancer (Sung et al., 2009). In addition ZER was reported to effectively suppress mouse colon and lung carcinogenesis through multiple modulatory mechanisms (Kim et al., 2009).

However, to the best of our knowledge, no chromatographic method has been described in literature for ZER determination in human plasma as well as other biological fluids. Here, we present for the first time an easy, HPLC method for determination of ZER in human plasma which requires minor laboratory efforts. This method is one step plasma protein precipitation followed by injection into HPLC with Photodiode Array (PDA) detector which is available in most analytical laboratories. The method will reduce the experimental work to almost 50%.

MATERIALS AND METHODS

Chemicals

ZER was extracted from *Zingiber zerumbet* rhizomes obtained from the wet market in Kuala Lumpur, Malaysia. The purity of the extracted ZER was > 98%. Structurally similar, α-Humulene (HUM) (Figure 1 (A) and (B)) was used as internal standard (IS) and was purchased from Sigma Chemical Co. (Street Louis, MO, USA). HPLC grade acetonitrile, methanol and potassium dihydrogen orthophosphate monobasic were purchased from Fisher Scientific (USA). Ultra pure water was used throughout the experiment.

Standard preparation and quality control

Stock and working solutions of ZER and HUM (100 µg/ml) was prepared by dissolving in acetonitrile. Standard solutions of ZER in human plasma were prepared by spiking the diluted stock solution, to give the final concentrations of 2, 4, 6, 8, 10 and 15 µg/ml. The internal standard HUM solution 50 µg/ml was also prepared.

HPLC apparatus and chromatographic conditions

HPLC system consist of Alliance separation module model e2695 with PDA detector, the signals were processed by Empower™ software (Waters, Milford, MA, USA). The mobile phase composed of acetonitrile: methanol: 0.01 M potassium dihydrogen orthophosphate (25: 55: 20). The analytical column used was C_{18} (Symmetry, 250 X 4.6 mm ID, 5 µm particles size) at an ambient temperature. The elute was monitored by PDA detector at a flow rate of 1 ml /min in wavelength of 254 nm.

Sample preparation

Ten microliters of internal standard solution 50 µg/ml were added to 150 µl plasma sample in microcentrifuge tube. The tube was vortex for 1 min and 200 µl of acetonitrile was added and vortexed for 2 - 4 min to precipitate the plasma protein, then the sample was centrifuged at 10000 rpm for 15 min. The supernatant was withdrawn in 300 µl insert vials and 10 µl was injected into HPLC system.

Validation procedure

The validation parameters obtained were specificity, linearity, accuracy, precision, stability and robustness. The method was validated according to FDA guidelines.

Specificity

Specificity was obtained by comparing chromatograms of 6 different batches of blank plasma obtained from 6 different subjects and plasma samples spiked with ZER and HUM.

Linearity and lower limit of quantification (LOQ)

Calibration plots were constructed from blank plasma spiked with HUM and six concentrations of ZER (2 – 15 µg/ml). The linearity of each calibration curve was determined by plotting the peak area ratio(y) of ZER to HUM vs the nominal concentration (x) of ZER. The calibration curves were constructed by weighted (1/x) least – square linear regression method. The lower limit of quantitation (LOQ) is the lowest concentration of the analyte that can be determined with acceptable precision and accuracy.

Precision and accuracy

The intra-day and inter-day precision and accuracy of the method were determined by percent coefficient of variation (% CV) and percentage relative error (% RE) respectively, according to the reported guidelines (FDA guidance for industry, 2001; Shah et al., 2000). Quality Control (QC) samples containing 3, 7 and 12 µg/ml concentrations were spiked for determination of precision and

Table 1. Intra- and Inter-day precision and accuracy of ZER in human plasma.

Nominal Conc. (µg/ml)	Calculated Conc. (µg/ml)		C.V.%		% Error	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
3	2.98	2.85	3.47	7.46	-0.62	-4.89
7	7.13	6.77	2.43	5.71	1.79	-3.26
12	12.27	11.87	2.81	8.11	2.28	-1.05

accuracy. Three replicates at each concentration were processed as described in the sample preparation on day 1, 2, 3 and 15 to determine intra-day and inter-day precision and accuracy. The % RE values were calculated by the following equation:

[(CC - AC) / AC] X 100

Where: CC = Calculated concentration, AC = Added concentration

Recovery

The recovery of ZER was determined for QC samples at the three samples pools 3, 7, 12 µg/ml. Three replicates of each QC sample were treated as mentioned in the sample preparation previously and injected into HPLC system. The extraction recovery was calculated by the following equation:

Recovery = [(peak area after extraction) / (peak area after direct injection)] X 100

Stability

The stability studies were carried out at the three different concentrations of the QC values (3, 7, 12 µg/ml) stored at room temperature (bench top stability) was evaluated for 24 h and compared with freshly prepared extracted samples. Freeze and thaw stability for three cycles was determined at the same concentrations (QC), by thawing at room temperature for 2 - 6 h and then refreezing at -20°C for 12 - 24 h. The long term stability of ZER at the same QC values was assessed by carrying out the experiment after 15 days of the storage in -30°C. The concentration of ZER after each storage period was related to the initial concentration as determined for the samples that were freshly prepared.

Robustness

The robustness of a method is its capability to remain unaffected by small, but deliberate variations in method parameters. The effect of percent organic strength as well as the buffer on resolution was studied by varying acetonitrile from -1 to +1%, methanol from -2 to +2% and 0.01 M KH_2PO_4 from -1 to +1% while the other mobile phase components were remained constant as stated in chromatographic condition. The flow rate was changed by ± 0.2 units while the other mobile phase components were remained constant as stated in chromatographic condition. The column temperature was also studied in such a way that the temperature was studied at ambient, 25 and 30°C.

RESULTS AND DISCUSSION

The chromatographic conditions and sample preparation for the proposed analytical procedure were optimized to be suitable for preclinical pharmacokinetic studies. The main challenge here is to use the smallest plasma volume as much as you can in order to avoid the risk of contamination and poor recovery as well. Unlike solid phase extraction for purification of plasma samples for HPLC analysis that needs more plasma volumes (Cao et al., 2008), protein precipitation by acetonitrile is simple, not tedious and less risky for the analyst. As shown in the chromatograms of human plasma (Figure 2), the retention times were 4.5 and 8.89 min for HUM and ZER respectively, with a total run time of less than 10 min. The analytical process of ZER and HUM were resolved with good symmetry. No interfering peaks were observed in individual blank plasma at the retention time of ZER and HUM, thus confirming the specificity of the method. System suitability parameters for the method were as follows: the theoretical plates of ZER and HUM were 8530, 4608, respectively. Tailing factors was less than 1.6 for both ZER and HUM and resolution between ZER and HUM was 11.6.

The peak area ratio of ZER to HUM was used for the quantification of ZER in human plasma samples. Figure 3, the mean of five calibration curves made over a period of 14 days, each calibration curve originating from a new set of extraction. The mean calibration curve was linear in the concentration range of 2 - 15 µg/ml and equation of the six points was $y = 0.1948X – 0.0862$ with correlation coefficient (r) of 0.9978. The accuracy and precision were evaluated with QC samples at concentrations of 3, 7 and 12 µg/ml. The intra-day precision (expressed as percentage error RE) was deter-mined by the analysis of three replicates of QC samples at three different concentrations. The inter-day accuracy and precision were determined on three different days and the results were shown in Table 1. The intra-day and inter-day of the QC values were satisfactory with C.V% less than 10% and accuracy with RE within ±3%.

The lower LOQ was calculated by determining the concentration of four spiked calibration standards and was found to be 2 µg/ml for ZER in human plasma with CV of less 20% and the accuracy of 88 - 112%. The limit of detection (LOD) was determined to be 1.5 µg/ml based on signal to noise ratio (s/n) ratio of 3:1. The extraction recovery was determined by standard addition at three different concentrations (3, 7 and 12 µg/ml) for ZER and one concentration (10 µg/ml) for HUM and was found to be 95 ± 3, 97 ± 1 and 96 ± 2 for ZER and 92 ± 3 for HUM, Table 2. The recovery of ZER using the described

Table 2. ZER recovery and accuracy of the assay.

Conc.(µg/ml)	Absolute recovery				Accuracy (%)		
	Conc. (µg/ml) (Mean ± SD)	Mean (%)±SD (n = 3)	Range (min - max)	C.V.%	Mean ±SD (n = 3)	Range (min - max)	C.V.%
3	2.71 ± 0.24	95.44 ± 3.4	93 - 99	4	94 ± 6	90 - 100	6
7	6.69 ± 0.12	97.00 ±1.2	89 - 92	1	97 ± 3	94 - 101	3
12	11.39 ± 0.20	92.11 ± 3.1	90 - 97	3	93 ± 4	92 - 99	4

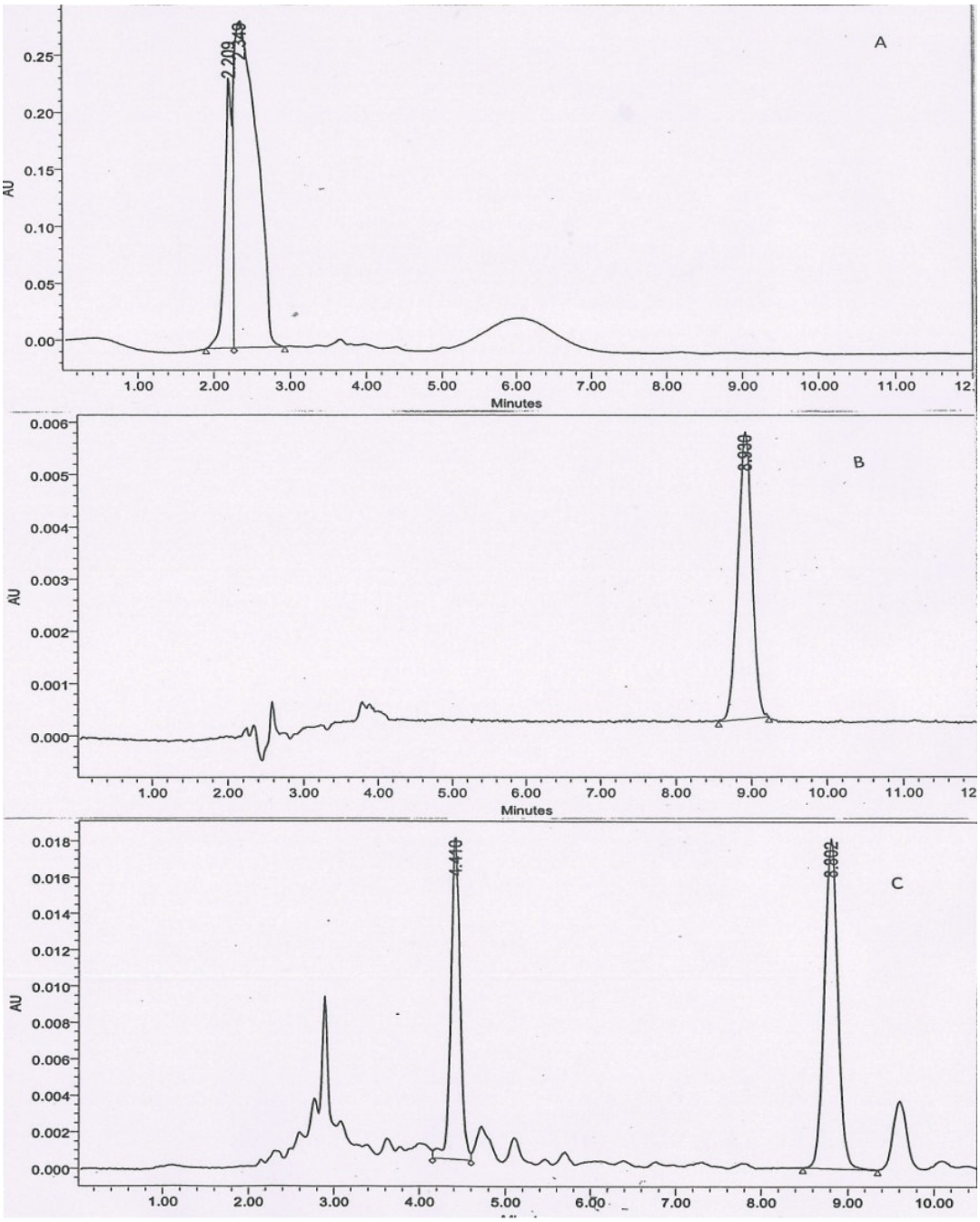

Figure 2. Typical chromatograms of human plasma (drug free) (A), blank plasma spiked with ZER (2 µg/ml) (B) and human plasma spiked with 10 µg/ml of ZER and 10 µg/ml of HUM.

Table 3. Stability of ZER in three different concentrations at three different conditions (bench top, freezing and thawing cycles and long term storage).

Stability	Spiked Conc. (µg/ml)	Calculated Conc (µg/ml) Mean ±SD(n = 3)	Average %	% C.V.
Bench Top[a]	3	2.99 ± 0.07	96	2
	7	6.64 ± 0.50	95	8
	12	10.27 ± 0.14	88	1
Freeze and Thaw[b]	3	2.97 ± 0.04	97	1
	7	6.63 ± 0.57	94	9
	12	10.79 ± 0.70	90	3
Long Term[c]	3	NT[d]	NT[d]	NT[d]
	7	6.5 ± 0.30	93	5
	12	10.4 ± 0.70	87	7

[a]After 24 h at room temperature.
[b]After three freeze thaw cycles.
[c] After 15 days at -30°C.
[d] Not tested.

Table 4. The robustness data of the assay method.

Parameter	Modification	RT (min)		Tailing Factor		Plates		Resolution
		ZER	HUM	ZER	HUM	ZER	HUM	
Mobile phase ratios MeOH:ACN:Buffer	53:26:21	9.27	4.41	1.2	1.3	7511	3844	13.80
	55:25:20	8.75	4.39	1.5	1.0	6615	3721	12.00
	57:24:19	10.09	4.9	1.3	1.3	12723	4624	14.60
Flow rate (ml/min)	0.80	11.09	5.54	1.2	1.0	10404	5929	10.06
	1.00	8.75	4.39	0.7	1.4	9526	6615	9.00
	1.20	7.36	3.69	1.3	1.5	6790	4624	11.56
Column Temp. (°C)	Ambient	8.89	4.44	1.5	0.8	6833	3844	12.40
	25	8.69	4.38	1.3	1.0	9526	3721	13.33
	30	8.19	4.22	1.0	1.2	6834	4547	13.18

method was consistent and efficient.

Analysis of the stock solution was performed at 200 µg/ml and after storage for two weeks at 4°C and at room temperature for 24 h, more than 99% of ZER remained unchanged, based on the peak areas in comparison with freshly prepared solution of ZER (200 µg/ml). Thus, ZER in standard solution was stable at least 14 days when store at 4°C and for 24 h at room temperature. Bench top stability of ZER was investigated at the concentration of 3, 7 and 12 µg/ml and the results revealed that the ZER in plasma was stable for at least 24 h with average percentage of 96, 95 and 88% respectively. Repeated freezing and thawing (three cycles) of plasma samples spiked with ZER at three levels (3, 7, 12 µg/ml) showed a mean percentage concentration of 97, 94 and 90%, respectively. Long term stability of ZER in plasma at -30 was also performed after 15 days of storage at three levels (3, 7, 12 µg/ml), which showed mean percentage concentration of (non-tested, vial was broken), 93, 87% respectively. The results of the stability study was presented in Table 3 and indicated that ZER was stable in the studied conditions.

The robustness results are shown in Table 4. It can be seen that the chromatographic conditions in accordance with established value (Shah et al., 2000). A change in mobile phase composition (acetonitrile 25 ± 1% v/v), (methanol 55 ± 2% v/v), (0.01 M KH_2 PO_4 20 ± 1% v/v), flow rate (1 ± 0.2 units) and column temperature (ambient, 25 and 30°C) had no impact on chromatographic performance. The tailing factor for both ZER and HUM was less than 2 and the two analytes (ZER and HUM) were well separated under the conditions carried out. The resolution between ZER and HUM was ranged 9.0 - 14.6. Considering the result of modifications in the system suitability parameters and the specificity of the method, it could be concluded that the method conditions are robust.

Conclusion

The newly developed HPLC method for determination of

ZER in micro-volumes human plasma (| 1.5 ml) was found to be simple, accurate, specific and robust. The method consisted of one step plasma protein precipitation using acetonitrile, followed by chromatographic separation in photodiode detector. No interference peaks were observed at the retention time of both ZER and HUM. This method could therefore be recommended for *in vivo* analysis of ZER for preclinical pharmacokinetic study and may also be applied for the estimation of ZER in other biological fluids such as serum and tissues after partial validation.

ACKNOWLEDGMENTS

The authors wish to express sincere appreciation to the University Putra Malaysia for the financial support of this research (RUGS 91143) and to the Laboratory of immunotherapeutic and vaccines (LIVES) Institute of Bioscience, UPM for providing technical guidelines. The authors gratefully acknowledge the competent technical assistance of Mr. Mohd. Hanif. MD. Arshad.

REFERENCES

Abdul AB, Al-Zubairi AS, Tailan ND, Wahab SIA, Zain ZNM, Ruslay S, Syam MM (2008). Anticancer Activity of Natural Compound (ZER) Extracted from *Zingiber zerumbet* in Human HeLa Cervical Cancer Cells. Int. J. Pharmacol. 4(3): 160-168

Cao YG, Zhang M, Yu D, Shao JP, Chen YC, Liu XQ (2008). A method for quantifying the unstable and highly polar drug nafamostat mesilate in human plasma with optimized solid-phase extraction and ESI-MS detection: more accurate evaluation for pharmacokinetic study. Anal. Bioanal. Chem. 391: 1063-1071.

FDA Guidance for Industry (2001). Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, MD, 2001.

Kim M, Miyamoto S, Yasui Y, Oyama T, Murakami A, Tanaka T (2009). ZER, a tropical ginger sesquiterpene, inhibits colon and lung carcinogenesis in mice. Int. J. Cancer 124(2): 264-271.

Kitayama T, Okamoto T, Hill RK, Kawai Y, Takahashi S, Yonemori S, Yamamoto Y, Ohe K, Sawada S (1999). Chemistry of ZER 1. Simplified isolation, conjugate addition reactions, and a unique ring contracting transannular reaction of its dibromide. J. Org. Chem. 64:2667-2672.

Kitayama T, Yamamoto K, Utsumi R, Takatani M, Hill RK, Kawai Y, Sawada S, Okamoto T (2001). Chemistry of ZER 2. Regulation of ring bond cleavage and unique antibacterial activities of ZER derivatives. Biosci. Biotechnol. Biochem. 65: 2193-2199.

Matthes HWD, Luu B, Ourisson G (1980). Cytotoxic components of *Zingiber zerumbet*, *Curcuma zedoria* and *C. domestica*. Phytochemist, 19:2643-2650.

Murakami A, Takahashi D, Kinoshita T, Koshimizu K, Kim WH Kim HW, Yoshihiro A, Nakamura Y, Jiwajinda S, Terao J, Ohigashi H (2002). ZER, a Southeast Asian ginger sesquiterpene, markedly suppresses free radical generation, proinflammatory protein production and cancer cell proliferation accompanied by apoptosis: The alpha, beta-unsaturated carbonyl group is a prerequisite. Carcinogenesis, 23: 795-802.

Murakami A, Takahashi M, Jiwajinda S, Koshimizu K, Ohigashi H (1999). Identification of ZER in *Zingiber zerumbet* Smith as a potent inhibitor of 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein–Barr virus activation. Biosci. Biotechnol. Biochem. 63: 1811-1812.

Murakami A, Tanaka T, Lee JY, Surh YJ, Kim HW, Kawabata K, Nakamura Y, Jiwajinda S, Ohigashi H (2004). ZER, a sesquiterpene in subtropical ginger, suppresses skin tumor initiation and promotion stages in ICR mice. Int. J. Cancer 110: 481-490.

Ohe K, Miki K, Yanagi S, Tanaka T, Sawada S, Uemura S (2000). Selective conjugate addition to ZER and transannular cyclization of its derivatives. J. Chem. Soc. Perkin Trans. 1: 3627-3634.

Sakinah SA, Handayani ST, Azimahtol LP (2007). ZER induced apoptosis in liver cancer cells via modulation of Bax/Bcl-2 ratio. Cancer Cell Int. 7: 4.

Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, Mc Gilveray IJ, Mc Kay G, Miller KJ, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT, Yacobi A (2000). Bioanalytical method validation- a revisit with a decade of progress. Pharm. Res. 17: 1551-1557.

Somchit N, Nur-Shakirah MH (2003). Anti inflammatory property of ethanol and water extracts of *Zingiber zerumbet*. Indian J. Pharmacol. 35: 181-182.

Sung B, Murakami A, Oyajobi BO, Aggarwal BB (2009). ZER abolishes RANKL-induced NF-κB activation, inhibits osteoclastogenesis, and suppresses human breast cancer induced bone loss in athymic nude mice. Cancer Res. 69: 1477-1484

Tanaka T, Shimizu M, Kohno H, Yoshitani S, Tsukio Y, Murakami A, Safitri R, Takahashi D, Yamamoto K, Koshimizu K, Ohigashi H, Mori H (2001). Chemoprevention of azoxymethane-induced rat aberrant crypt foci by dietary ZER isolated from *Zingiber zerumbet*. Life Sci. 69: 1935-1945.