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ORIGINAL ARTICLE

Assessment of cell cycle phase-specific effects of zerumbone on mitotically synchronous surface cultures of *Physarum polycephalum*

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Abstract Zerumbone, a natural cyclic sesquiterpene, has been the focus of recent research as it has been found to exhibit selective toxicity towards cancer cells compared to normal cells. Studies on the cell cycle phase-specific effects of this interesting compound, however, remain sparse. Hence, concentration and time-dependent effects of zerumbone were evaluated employing a suitable model system, the naturally synchronous surface cultures of *Physarum polycephalum.* Zerumbone treatment in S, early, and late G2 phases resulted in G2 arrest. Early G2 phase exhibited the highest sensitivity ($P<0.001$) to the compound. Protein profiles showed a complete inhibition of cyclin B1 expression following zerumbone treatment. Furthermore, FACS and comet analysis revealed that zerumbone inhibited DNA synthesis ($P<0.001$) without being genotoxic at the concentrations tested. Differential display of mRNA showed distinct zerumbone-induced variations in transcript profiles, an analysis of which suggested a likely link between cellular networks involving stress-related gene expression and G2 arrest in *P. polycephalum.*

Keywords *Physarum polycephalum* · Zerumbone · G2 arrest · G2 release · Cyclin B1 · DDRT-PCR

Introduction

Plants have been widely exploited for health benefits in tribal and folklore medicine since ancient times. It is now well known that the therapeutic benefits emanate from phytochemicals contained therein which possess diverse, pharmacologically active compounds (Dillard and German 2000). The family Zingiberaceae comprising of rhizomatous medicinal and aromatic plants have been a rich source of compounds of phytomedical interest. *Zingiber zerumbet* (L) Smith, known as wild ginger, originally an Indian plant widely distributed in Bangladesh, Nepal, Sri Lanka, and Malaysia (Anon 1976) has been traditionally used for treating various ailments. These include stomach ache, ear inflammation, swelling, sores, loss of appetite, roundworm infestations, diarrhea, and fever (Pushpangadan and Atal 1984; Bhuiyan et al. 2008). The volatile oil from its rhizomes has also been used to relieve rheumatic pain (Bhuiyan et al. 2008). Parts of the plant such as young stems and inflorescence are edible and used in traditional cooking (Kankuri et al. 1999). A cyclic 11-membered monosesquiterpene, first described by Varier (1945), subsequently named as "Zerumbone" (Parihar and Dutt 1950) was first isolated by Dev (1960) from the rhizomes of *Z. zerumbet.*

Zerumbone possesses tremendous therapeutic potential since it shows a diverse range of biological activities such as antibacterial, antiviral, antimutagenic (Santosh Kumar et al. 2013; Dai et al. 1997), antinociceptive (Sulaiman et al. 2010a), anti-inflammatory (Sulaiman et al. 2010b), antiulcer, antihyperglycemic, antiplatelet aggregation (Yob et al. 2011), antioxidant, and hepatoprotection (Fakurazi et al. 2009). At high doses, zerumbone is reportedly cytotoxic to human peripheral blood lymphocytes but not clastogenic (Al-zubairi et al. 2010) while exerting minimal effects on growth of normal human dermal and colon fibroblasts (Murakami et al. 2002). Recent reports have also highlighted its anticancer and antitumor activities (Kirana et al. 2003). Zerumbone-induced apoptosis in different cell lines has been shown to be mediated through the involvement of several proteins such as Bax-Bak (Sehrawat et al. 2012), p53 (Zhang et al. 2012), interleukin-6

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(Abdelwahab et al. 2012), fas (Xian et al. 2007), tumor necrosis factor-related apoptosis-inducing ligand (Yodkeeree et al. 2009), Gli/bcl2 (Sun et al. 2013), IKKα, Akt, and FOXO1 (Weng et al. 2012). It has also been reported to cause G2/M cell cycle arrest in HeLa (Abdelwahab et al. 2012), HepG2 (Muhammad Nadzri et al. 2013), HUVEC, HL-60, NB4 (Xian et al. 2007), MCF-7, and MDA-MB-231 cells (Sehrawat et al. 2012). However, reports on the phase-specific and dose-dependent effects of zerumbone on cell cycle per se were found lacking. Hence, an attempt has been made to evaluate its effects on the cell cycle employing a suitable model system derived from the lower eukaryotic slime mold, *Physarum polycephalum*.

The vegetative phase of *P. polycephalum*—termed macroplasmodia—is a syncytium that can be grown to a large size as a "giant cell" in which more than 10^8 nuclei divide synchronously (Guttes and Guttes 1964; Rusch et al. 1966) wherein rhythmic intranuclear mitoses occur every 8–10 h, which can be monitored using phase microscopy (Guttes et al. 1961) (Fig. 1). G1 phase being absent, the end of telophase marks the beginning of S phase which roughly lasts for about one third of the cell cycle followed by a protracted G2 phase (Nair 1995). Interestingly, the G2/M phase transition period is also notable since it is characterized by nucleolar migration toward the periphery of nuclear membrane (Guttes et al. 1961). G2 checkpoint assumes critical importance in the absence of G1 phase mimicking a situation akin to many cancers where the G1 checkpoint often becomes defunct. In the present study, the cell cycle modulatory effects of zerumbone on *Physarum* were evaluated in terms of changes in mitotic timings, nuclear DNA content, overall protein, and transcript profiles.

Materials and methods

Chemicals and reagents

Zerumbone, BCIP/NBT, DMSO, propidium iodide, TRI reagent, anchored primers, anti-cyclin B1, and anti-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris base, glycine, ethidium bromide, H_2O_2, and Triton X-100 were purchased from SRL Pvt. Ltd., Mumbai, India. Goat anti-rabbit IgG-ALP conjugate, agarose, arbitrary primers, DNase I, RNasin, dNTP mix, M-MLV reverse transcriptase, and taq DNA polymerase were procured from Genei, Bangalore, India. Positively charged nylon membranes were purchased from BDH laboratories supplies, England. All other chemicals and reagents used were of analytical grade.

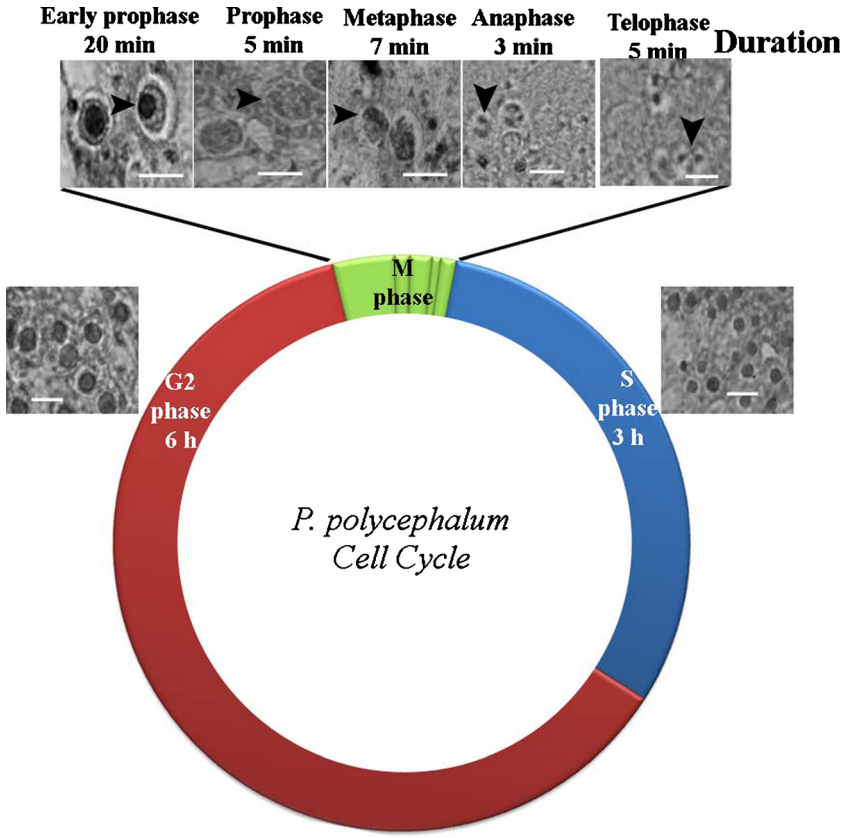

Fig. 1 *P. polycephalum* cell cycle duration and phase contrast micrographs of ethanol-fixed plasmodial smears showing representative nuclei from M, S, and G2 phases. Scale bars: 10 μm for all images

P. polycephalum culture

Microplasmodial suspension cultures of the McArdle Strain (M_3C VIII) of *P. polycephalum*, grown in the dark at 24–26 °C in semi-defined medium (SDM) (Daniel and Baldwin 1964), were fused to prepare mitotically synchronous surface (macro) plasmodia which were grown on filter papers, moistened with SDM, supported by glass beads in petri dishes (Guttes et al. 1961).

Zerumbone treatments and determination of mitotic stages

Each macroplasmodia was cut into equal-sized sectors. Experimental sectors were placed in SDM containing different concentrations of zerumbone. Control sectors received equivalent amounts of the solvent vehicle, DMSO. Ethanol-fixed smears prepared from macroplasmodial explants were employed to observe post-fusion mitoses (PFM) under a phase-contrast microscope taking metaphase as reference point to compute cell cycle delays (Holt 1980). All experiments were carried out between the II and III PFM to allow for sufficient growth (4–5 cm diameter). Cell cycle duration in control macroplasmodia was of 9–10 h. In a 10-h cycle, S phase which commences at the end of telophase lasts for about 3 h followed by G2 phase of about 6 h.

SDS-PAGE and Western blot analysis

For protein analysis, samples were taken from *Physarum* macroplasmodia treated with zerumbone at a concentration (50 μg/ml) which elicited the maximal differential mitotic delays with respect to S and G2 phases. Macroplasmodial disks (7 mm) were punched out and rapidly precipitated with five volumes of ice cold acetone at –20 °C for 10 min and centrifuged at 12,000*g* for 5 min. The dried control and zerumbone-treated protein samples were directly solubilized in the sample buffer, incubated in boiling water bath for 2 min, subjected to SDS-PAGE on 12.5 % Laemmli gels, and monochromatic silver staining (Hames 1990). The gels were photographed on AlphaImager 2,200 (USA) documentation system, and molecular weight of the polypeptides was determined using AlphaEaseFC software. The band of interest was sliced off the gel, and the protein was extracted into the elution buffer (50 mM ammonium bicarbonate, 5 % 2-mercaptoethanol, and 0.1 % SDS), precipitated with 20 % TCA, followed by ice cold acetone (Rosenberg 2005). The recovered protein was reconstituted in sample buffer for SDS-PAGE.

For Western blot analysis, the separated proteins were blotted onto positively charged nylon membranes and incubated in blocking buffer with 10 % skimmed milk in Tris-buffered saline (100 mM Tris-HCl, pH 7.5, 0.9 % NaCl) for 1 h. The membranes were incubated separately in polyclonal primary antibodies (anti-cyclin B1 and anti-actin) diluted with blocking buffer (1:600 for anti-cyclin B1; 1:1,000 for anti-actin) for 1 h at room temperature. Following five washes with Tris-buffered saline (TBS) for 15 min each, the membranes were incubated for 1 h in secondary antibody (goat anti-rabbit IgG-ALP conjugate) diluted with blocking buffer (1:2,000). Color visualization following a repeat of TBS washes was carried out using the chromogenic substrate BCIP/NBT (Ausubel et al. 1992).

Comet assay and flow cytometry of nuclear DNA

Being a syncytium, nuclear suspensions, prepared from control (at the time of prophase onset) and zerumbone-treated (G2 arrested) plasmodial sectors, were subjected to alkaline gel electrophoresis and FACS analysis as described earlier by us (Rajan et al. 2013). The nuclear comets were observed on a Leitz microscope (Dialux 20) and photographed using a Nikon D 60 SLR camera. Nuclei isolated from H_2O_2 (5 mM) treated macroplasmodia served as positive control. For FACS analysis, nuclear suspensions were washed with ice cold PBS, fixed in 70 % ice-cold ethanol and finally resuspended in 500 μl staining solution containing propidium iodide. DNA content analysis was done according to manufacturer's protocol on BD FACS Aria™ using BD FACS Diva software version 5.0.2.

Transcript profiling by differential display PCR

Isolation of total RNA

Total RNA was isolated from macroplasmodia using TRI reagent according to manufacturer's instructions. RNA was dissolved in sterile RNase-free water and quantified by spectrophotometry. For DNase I treatment, a 50 μl reaction containing 60 μg of total RNA, 5 μl 2.5× of DNase I buffer, 2.5 μl RNasin of 40 U/μl, 20.3 μl of RNase free DNase I (1,000 U/ml), and 12 μl of 25 mM $MgCl_2$ was incubated for 30 min in a 37 °C dry bath. RNA was then purified by phenol-CIA method and precipitated with ethanol. Following centrifugation, RNA pellet was resuspended in nuclease-free sterile water to give a final concentration of 0.5 μg/μl.

First strand cDNA synthesis and DD-PCR amplification

For first strand cDNA synthesis, a 10-μl reaction containing 1 μl of 10 mM dNTP mix, 1 μl of respective oligo-dT anchored primers (AP) (25 mM), and 1 μl of 0.5 μg/μl RNA was incubated at 70 °C for 10 min and placed on ice. To this, 2 μl of 10× M-MLV reverse transcriptase buffer, 1 μl of M-MLV reverse transcriptase, 0.5 μl of RNasin were added and made up to 20 μl with sterile nuclease-free water. The reaction mixture was incubated at 37 °C for 50 min and then

heated to 94 °C for 10 min. The reaction was stored on ice until the subsequent PCR reaction.

For DD-PCR, 25 μl reaction containing 1.25 μl of cDNA synthesis reaction, 2 μl of 2.5 mM mixed dNTPs, 2.5 μl of 10× Taq DNA polymerase buffer, 2.5 μl of 25 mM anchored oligodT primer (AP), 4 μl of 6.25 mM 10-mer arbitrary primer (RFu), and 0.3 μl of Taq DNA polymerase (3 U/μl) was used for PCR amplification as follows: 94 °C for 5 min, 35 °C for 2 min, and 72 °C for 2 min (initial cycle); 94 °C for 1 min, 35 °C for 2 min, and 72 °C for 1 min (40 cycles); 94 °C for 1 min, 35 °C for 2 min, and 72 °C for 10 min (final extention). PCR products were run on 1.2 % agarose gel and stained with ethidium bromide (Boschi and Vergara 1998). The bands of interest were excised using a sterile surgical blade and eluted for reamplification using Fermentas (Genetix Biotech Asia) gel extraction kit. The reamplified PCR products were checked on agarose gels once again to confirm their molecular weights and a selected few of these validated products were sequenced (SciGenom Labs Pvt. Ltd., Kochi, Kerala).

Statistical analysis

The data are expressed as the mean ± SD from three independent experiments. Results were analyzed for significance by one-way ANOVA using SPSS software version 16.0. Differences with $P<0.05$ were considered significant. Asterisks were used to identify the level of significance (*$p\leq0.05$, **$p\leq0.01$, and ***$p\leq0.001$).

Results

Effect of zerumbone on cell cycle of *P. polycephalum*

Zerumbone treatments on *P. polycephalum* showed a concentration and phase-specific effect on the cell cycle (Fig. 2). In early G2 phase treatments at concentrations ranging between 25 and 100 μg/ml, mitotic delays observed ranged from about 1 to 2.65 h, equivalent to about 10 to 30 % of the total cell cycle duration ($p<0.001$). Though S, early, and late G2 phase treatments elicited G2 arrest with a distinct concentration dependence, early G2 phase exhibited the highest sensitivity ($P<0.001$) to zerumbone. At the highest concentration tested, however, the delay pattern remained more or less the same as with that obtained at 50 μg/ml ($p<0.001$with mean difference=2.20667) except for a marginal increase with respect to S phase treatments.

Effect of zerumbone on protein expression in *P. polycephalum*

The results of protein profiling of control and zerumbone-treated samples by SDS-PAGE are shown in Fig. 3. Two sets of samples for protein analysis were collected at different time points as shown in the treatment schedule (Fig. 3a). One set comprising of zerumbone-treated, G2-arrested samples (lane S, EG2 and LG2 - G2 arrest) were collected along with the control at the time of its prophase onset (lane C PFM III) (Fig. 3b). The second set comprised of samples collected at prophase onset following release from G2 arrest in zerumbone-treated macroplasmodia (lane S, EG2, and LG2-

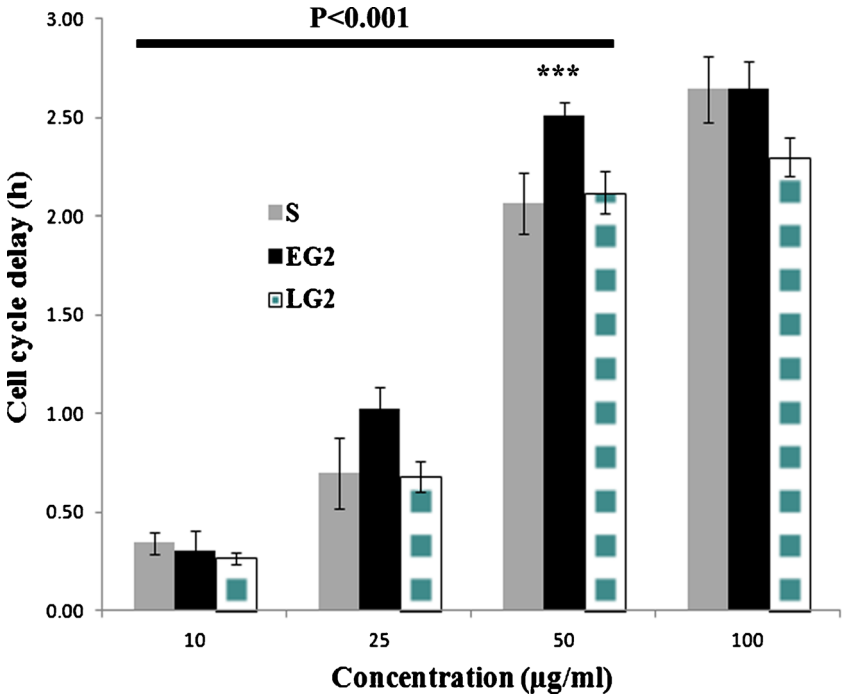

Fig. 2 Effect of zerumbone on *P. polycephalum* cell cycle—zerumbone treatments (10–100 μg/ml) of 3 h duration between II and III PFM at S phase and G2 phase (first 3 h represented as "early G2" and the rest as "late G2"). Data represents mean of three different experiments ± SD. The *error bar* represents SD. Values marked with *asterisks* represent significant delay ($p<0.001$)

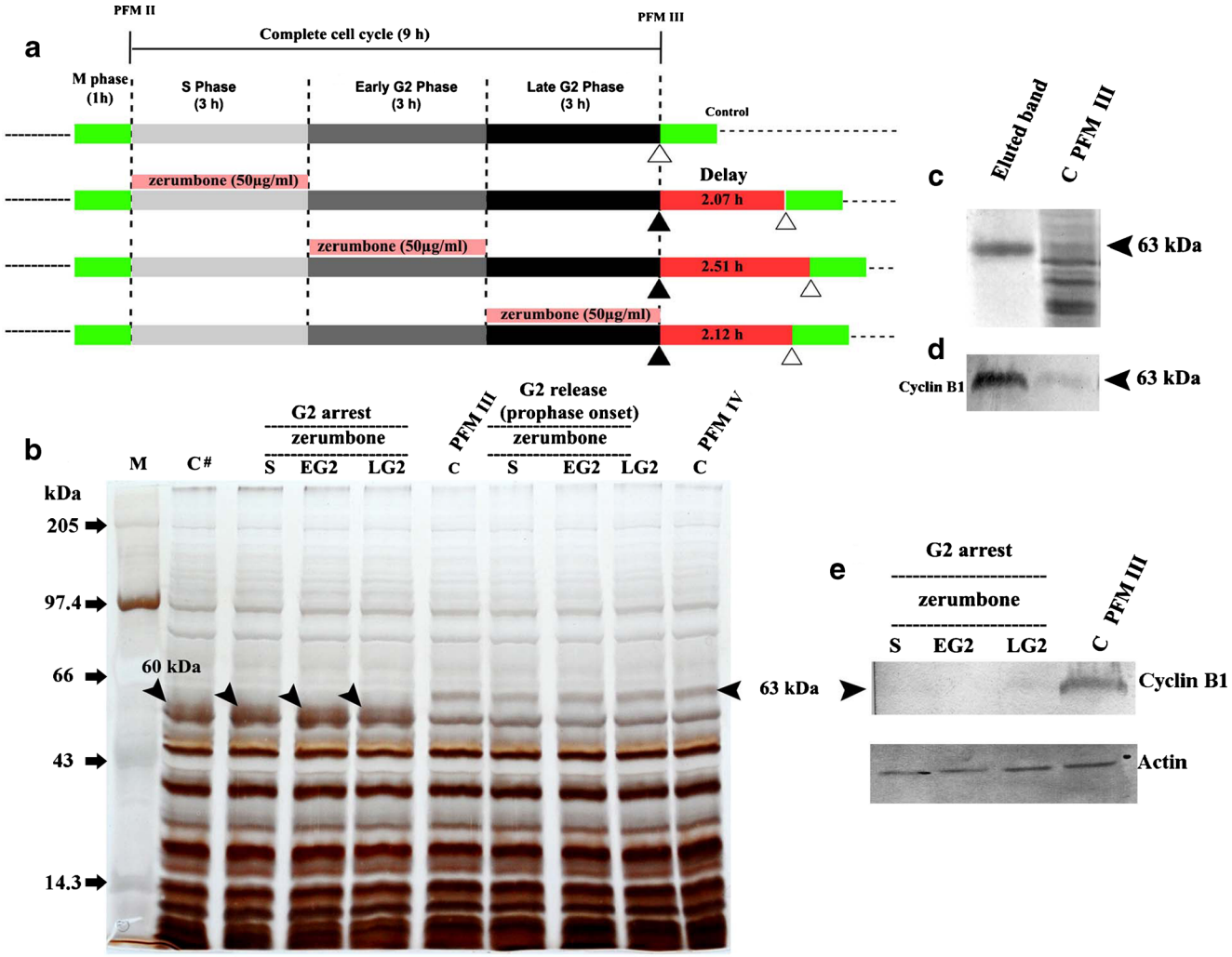

Fig. 3 Protein profiling by SDS-PAGE. **a** Schematic representation of zerumbone treatment on *P. polycephalum* and sample collection: PFM II and III second and third post-fusion mitosis, *black triangle* time at which G2-arrested samples were collected, *white triangle* sample collected at the time of G2 release. **b** Variations in *Physarum* macroplasmodial protein profiles of zerumbone-treated (50 µg/ml), G2-arrested, G2-released, and control samples analyzed on 12.5 % (*w*/*v*) polyacrylamide silver-stained slab gels. ~15 µg of protein was loaded in each lane. **c** 63 kDa band was excised from gel, concentrated, and separated by SDS-PAGE. **d** 63 kDa band was immunostained with anti-cyclin B1 antibody. **e** Control and G2-arrested samples were immunostained with anti-cyclin B1 antibody

G2 release) along with the sample collected from the untreated control at this time point (lane C#, Fig. 3b).

A 63-kDa polypeptide observed in controls at the time of prophase onset (lane C PFM III) was conspicuously absent in zerumbone-treated G2-arrested samples taken from all cell cycle phases concomitant with the appearance of the 60-kDa polypeptide (lane S, EG2, and LG2-G2 arrest). Polypeptide of 63 kDa was again found to be present in zerumbone-treated samples at prophase onset following release from G2 arrest (lane S, EG2, and LG2-G2 release). At this time point, interestingly, this 63 kDa polypeptide was found to be absent in control samples (lane C#) wherein it reappeared during the subsequent prophase (lane C PFM IV).

Since the cyclically appearing 63 kDa polypeptide observed by us closely resembled the cyclin B1 (62 kDa) already reported in *P. polycephalum* (Li et al. 2005), the 63-kDa band was excised out of the gel, concentrated, separated by SDS-PAGE, and immunostained with anti-cyclin B1 (Fig. 3c, d). Our results confirmed that the 63-kDa polypeptide present during prophase onset was indeed cyclin B1, which was absent in all G2-arrested samples irrespective of the cell cycle phase at which zerumbone treatment was given (Fig. 3e).

Effect of zerumbone on nuclear DNA

Nuclei isolated from the control and zerumbone-treated samples were analyzed for DNA integrity by comet assay. The absence of comets in zerumbone-treated samples indicated that the compound was not genotoxic at the concentrations tested while nuclear comets were found to be induced in the positive control treated with 5 mM H_2O_2 (Fig. 4). For flow cytometric analysis, the main peak at fluorescence mode 50

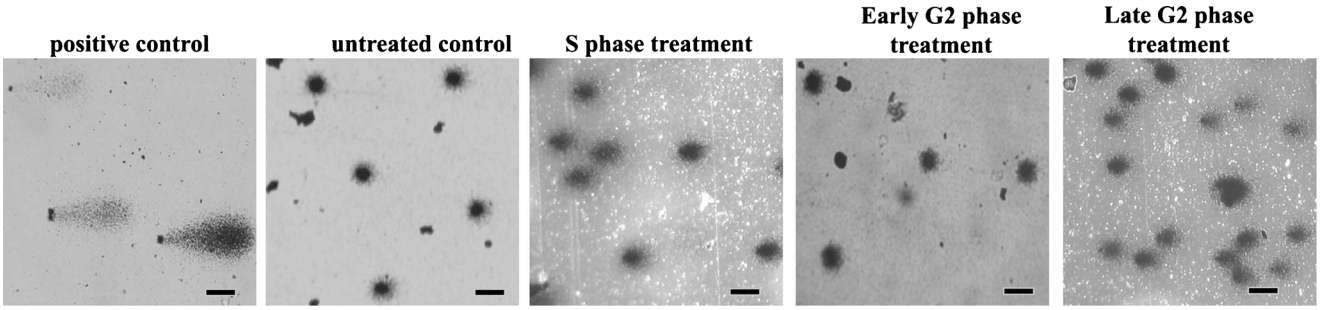

Fig. 4 Genotoxic effect of zerumbone on *P. polycephalum* was evaluated by Comet assay under alkaline condition. Scale bars: 10 μm for all images

representing ~70 % of synchronous nuclei was used for determination of DNA content. In comparison to control (at prophase onset), zerumbone treatment during S, early G2, and late G2 phase resulted in a progressive decline in DNA content by 34.7 ($p<0.001$ with mean difference 16.4), 25.3 ($p<0.001$ with mean difference 11.9667), and 12.6 % ($p<0.001$ with mean difference 5.9667), respectively (Fig. 5).

DDRT-PCR analysis of zerumbone-induced differential gene expression

DDRT-PCR was carried out to evaluate differentially expressed genes potentially involved in zerumbone-induced G2 arrest in *P. polycephalum* cell cycle. For this, samples treated with zerumbone at a concentration (50 μg/ml) which induced maximal differential mitotic delays were used along with the untreated control. We used 6 anchored primers and 6 arbitrary primers (Table 1) resulting in 36 primer combinations to analyze the differentially expressed genes in response to zerumbone treatment in early G2. Differentially expressed PCR products were separated on agarose gel (Fig. 6a) and analyzed essentially by the method of Venkatesh et al. (2005). The total number of bands obtained in control and treated were 195 and 181, respectively, averaging 63 bands, with respect to one primer combination (one arbitrary primer with six anchored ones) (Fig. 6b). Figure 7 depicts the results of the

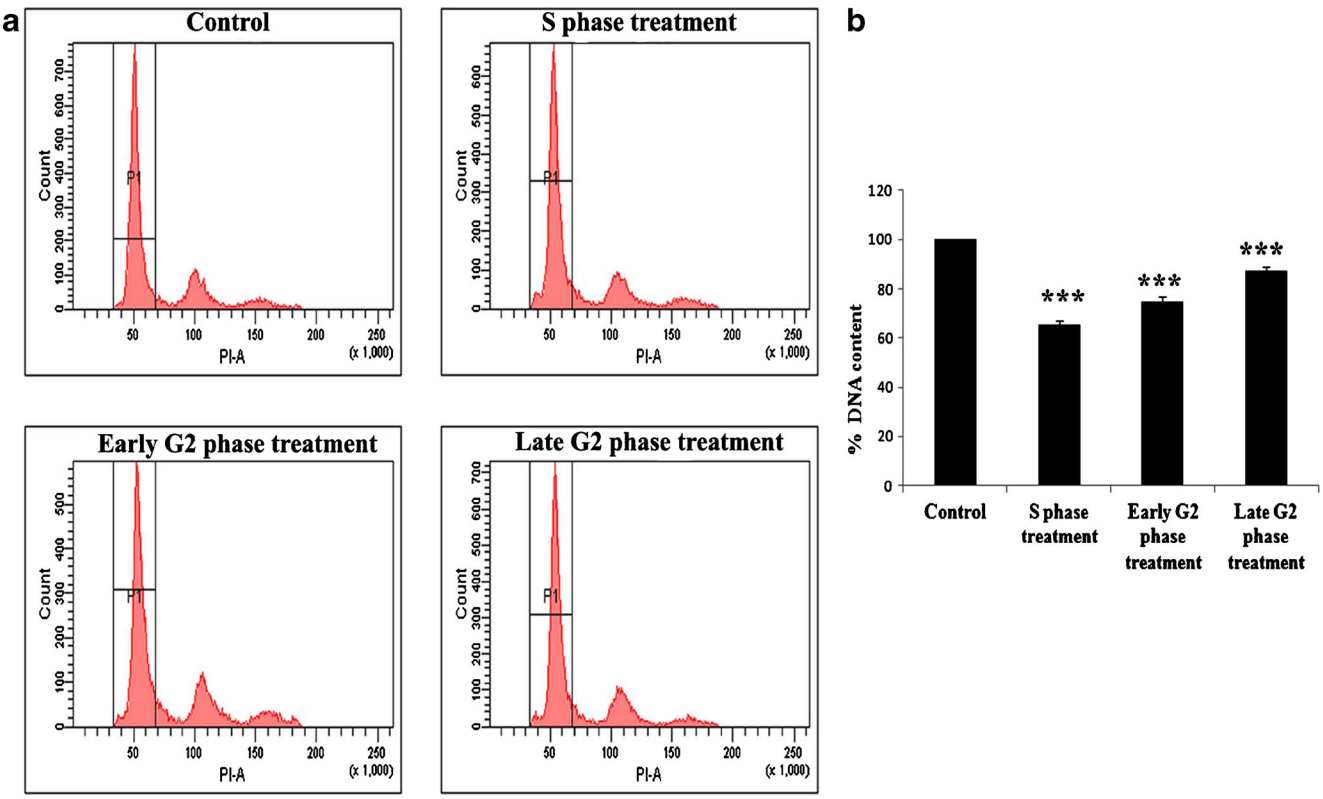

Fig. 5 Quantification of nuclear DNA content. **a** FACS analysis of propidium iodide stained synchronous nuclei isolated from *P. polycephalum* after zerumbone treatment during different cell cycle phases; **b** nuclear DNA content following zerumbone treatment with respect to controls. Data represents mean of three different experiments ± SD. The *error bar* represents SD. Values marked with *asterisks* represent significant changes in DNA content with respect to control ($p<0.001$)

Table 1 Primers used for DDRT-PCR

	No.	Name of primer	Sequence (5′–3′)
Arbitrary primer	1	RFu#1	CCTGGGCCAG
	2	RFu#2	CCTGGGCGAG
	3	RFu#3	CCTGGGCTGG
	4	RFu#4	CCTGGGCTAT
	5	RFu#5	CCTGGGCTTG
	6	RFu#6	CCTGGGCTAC
Anchored primer	1	AP1	TTTTTTTTTTTTAC
	2	AP2	TTTTTTTTTTTTAG
	3	AP3	TTTTTTTTTTTTGC
	4	AP4	TTTTTTTTTTTTGG
	5	AP5	TTTTTTTTTTTTCC
	6	AP6	TTTTTTTTTTTTCG

complete sets of bands produced by the various anchored primers used in this study. All of the six anchored primers used were found to produce significant differences. The size distribution of amplicons obtained with six primer combinations from control and zerumbone-treated samples is given in Fig. 8.

Results obtained following computation of induction factors showed that 38 amplicons were downregulated while 26 were upregulated and five showed twofold upregulation (Fig. 9a). Three amplicons detected in zerumbone-treated samples were found missing in the control while 15 missing in the treated samples were detected in control (Fig. 9b). A total of 21 amplicons (15, completely depressed and 6, newly induced) were sequenced. These amplicons were compared with the GenBank databases using the nucleotide BLAST search program. All of the selected 21 amplicons corresponded to EST or cDNA clones not yet fully characterized—9 matched with transcripts related to starvation stress library, 5 with non-normalized library, 1 with subtilisin-like protease B mRNA from *P. polycephalum* and 1 with *Dictyostelium discoideum* AX4 PHD zinc finger-containing protein (Table 2). The rest five products did not show any significant sequence similarity to previously reported genes deposited in the databases.

Discussion

A large number of natural products continue to be evaluated for their anticancer potential. There have been several reports

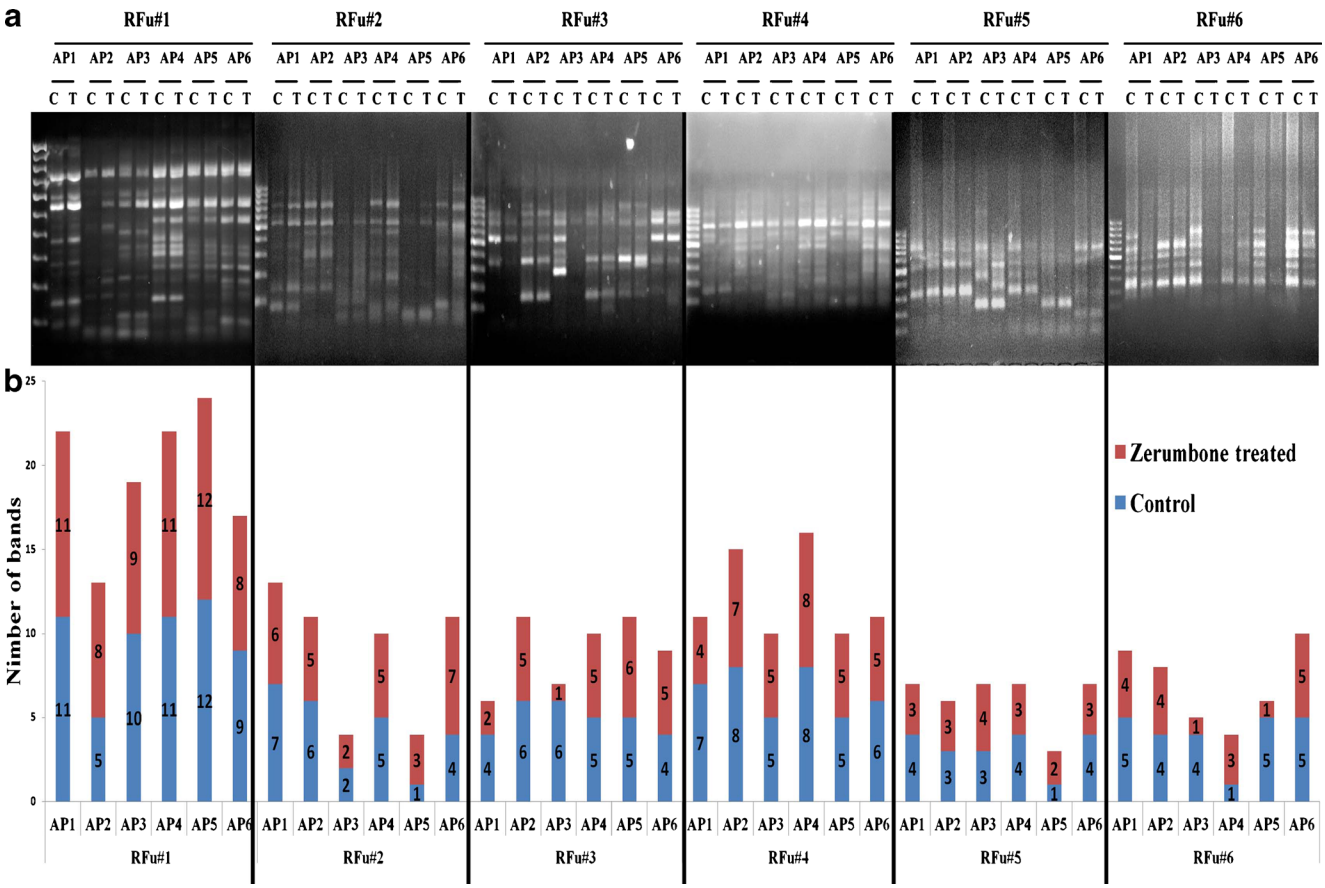

Fig. 6 Differential display analysis by DDRT-PCR. **a** Agarose gel images of DDRT-PCR products. *P. polycephalum* plasmodia were treated with zerumbone (50 μg/mL) at early G2 phase, where significant mitotic delay was observed. Thirty-six primer combinations were used for the amplification of RNA isolated from control (*C*) and treated (*T*) samples. **b** Number of bands produced by six arbitrary and six anchored primers

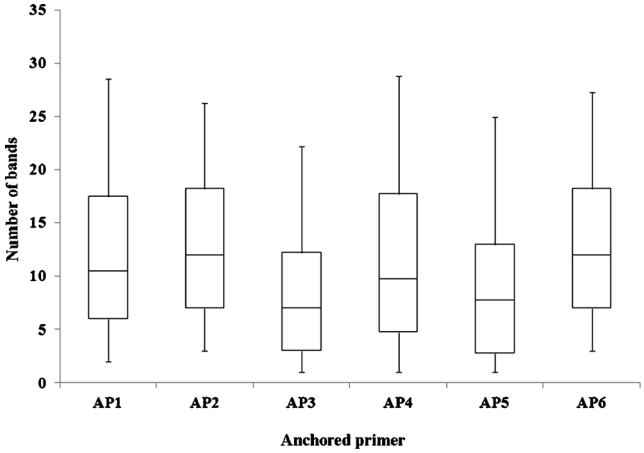

Fig. 7 *Box-and-whisker-plots* showing number of bands produced with anchored primers

on antitumor and anticancer activities of zerumbone in a variety of cancer cell lines. Interestingly, this compound has been shown to selectively target cancer cells compared to normal cells mediated by the induction of G2 arrest and apoptotic cell death in leukemia, ovarian, HL-60, NB4, and cervical cancer cells (Abdelwahab et al. 2011; Al-zubairi 2012; Huang et al. 2005; Sehrawat et al. 2012; Sun et al. 2013; Xian et al. 2007; Weng et al. 2012). However, our understanding of the cell cycle phase-specific effects of zerumbone remains inadequate. Almost all of these studies have been carried out on asynchronous cancer cell populations which preclude evaluations of dose-dependent effects of the compound under study on specific phases of the cell cycle. Induction of cell cycle synchrony by the use of physical and chemical agents is generally cumbersome due to difficulties in interpretation of results with regard to the effect of the compound under investigation. In this context, the present study on the macroplasmodial cultures of *P. polycephalum* assumes significance as this model system exhibits natural rhythmic synchronous mitotic divisions mimicking a cancer cell-like situation with G2 as the only functional checkpoint in the absence of G1 phase. A large number of nuclei in this acellular system progressing through extended S and G2 phases also facilitate a better understanding on concentration dependent effects which are also phase specific with respect to cell cycle.

The results of the present study distinctly show concentration dependent effects of zerumbone specific to S, early, and late G2 phases of *P. polycephalum* cell cycle. Irrespective of the cell cycle phase, zerumbone treatment was found to induce an effective G2 arrest. The early G2 phase was found to be most sensitive to zerumbone treatment. The differential effects of zerumbone on cell cycle were better evident at a dose of 50 μg/ml compared to that at 100 μg/ml. Exposure to curcumin during G2 phase was found to induce apoptosis in mammary epithelial carcinoma cells in a study employing time lapse video-micrography (Choudhuri et al. 2005). The causative mechanism underlying zerumbone-induced G2/M arrest has been shown to be mediated through the phosphorylation of chk1,chk2, Cdc25B, Cdc25C, and cdc2 and decline of cyclin B1 protein levels in HL-60, NB4, MCF-7, and MDA-MB-231 cells (Huang et al. 2005; Sehrawat et al.

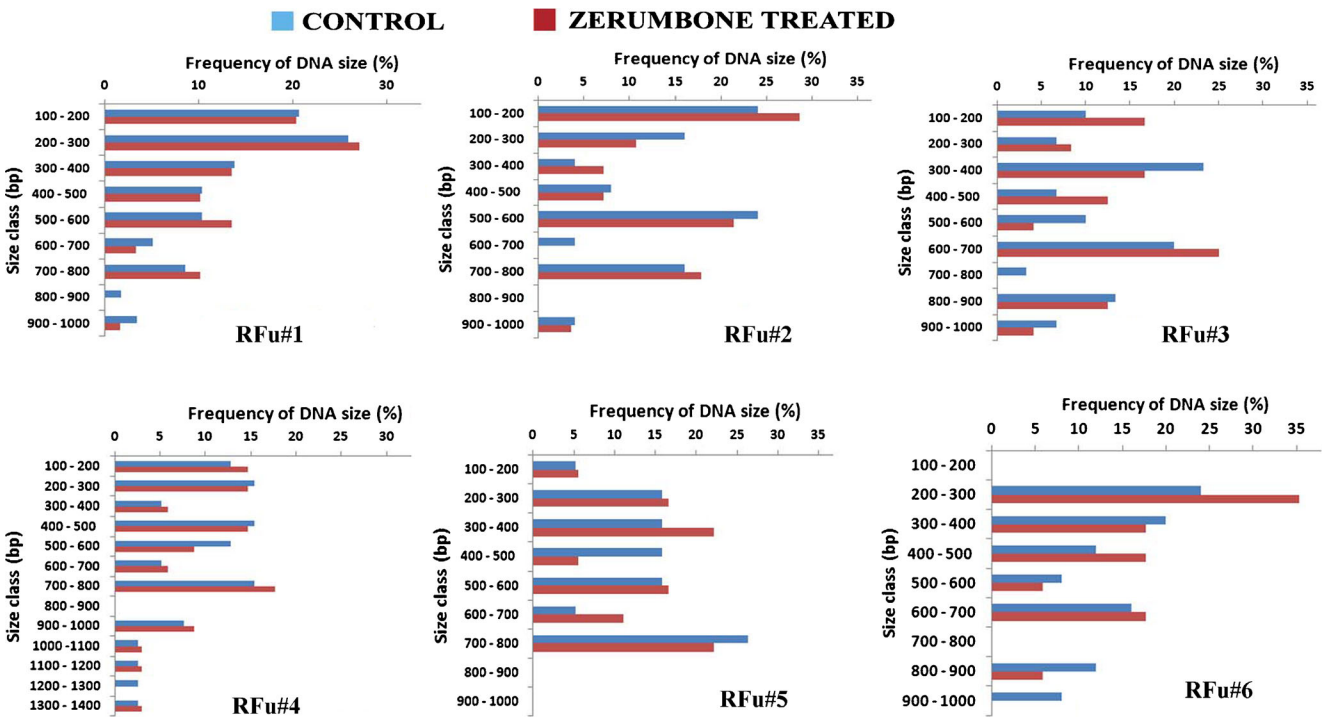

Fig. 8 Size distribution of amplified cDNA fragments

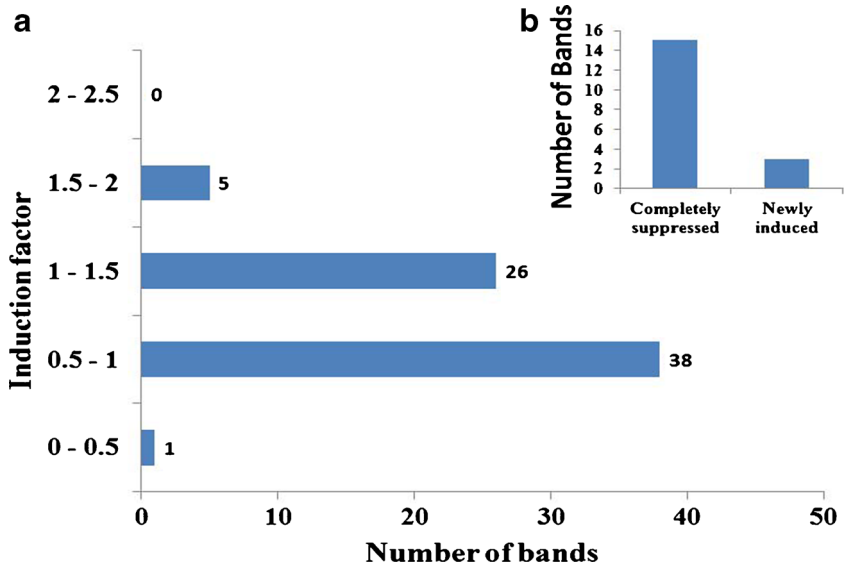

Fig. 9 Classification of cDNA bands. **a** Class assignment based on induction factor computation employing the ratio of control versus treated band intensities. **b** Zerumbone induced and suppressed classes

2012; Xian et al. 2007). The involvement of 63 kDa polypeptide in G2 arrest induced by the alkaloid extract from *Curcuma vamana* has already been reported by us in *P. polycephalum* (Rajan et al. 2013). This 63 kDa polypeptide was confirmed to be cyclin B1 based on immunostaining with anti-cyclin B1 antibodies in the present study. Treatment with zerumbone, irrespective of the cell cycle phase, namely, S, early, and late G2 resulted in inhibition of cyclin B1 expression. Since a functional cyclin B1-cdc2 complex is mandatory to traverse the G2/M checkpoint, the absence of cyclin B1 in zerumbone-treated samples should lead to G2 arrest. This indeed was found to be true as cyclin B1 was observed to reappear in protein profiles obtained in samples following release from G2 arrest. This is in line with the report by Li et al. (2005) that cyclin B1 in *P. polycephalum* makes it appear in S phase, gradually accumulates to peak at metaphase and then disappears at telophase (Li et al. 2003). In other words, our results clearly show that zerumbone treatment leads to G2 arrest by inhibition of cyclin B1 expression, albeit by a molecular mechanism which remains to be elucidated. The appearance of a 60-kDa polypeptide concomitant with the absence of cyclin B1, following zerumbone treatment also indicated that it could possibly have a role in *P. polycephalum* cell cycle regulation and this warrants further investigations.

Table 2 Differentially expressed genes following zerumbone treatment during early G2 phase of *P. polycephalum* cell cycle

No.	DDRT-PCR primer combination	BLAST result	Accession number	Fold change	Band size
1	RFu#1/AP1	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL571997.1	1.59	~350
2	RFu#1/AP2	*Dictyostelium discoideum* AX4 PHD zinc finger-containing protein	ref|XM_634951.1	1.55	~570
3	RFu#1/AP3	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL571997.1	1.61	~500
4	RFu#2/AP1	Non-normalized library—*P. polycephalum* cDNA	gb|EC758413.1	1.67	~600
5	RFu#3/AP3	Non-normalized library—*P. polycephalum* cDNA	gb|EC757837.1	Completely depressed	~370
6	RFu#3/AP3	*P. polycephalum* subtilisin-like protease B mRNA, partial cds	gb|DQ407661.1	Completely depressed	~690
7	RFu#3/AP3	Non-normalized library—*P. polycephalum* cDNA	gb|EC758413.1	Completely depressed	~430
8	RFu#3/AP3	Non-normalized library—*P. polycephalum* cDNA	gb|EC758707.1	Completely depressed	~700
9	RFu#5/AP1	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL571997.1	Completely depressed	~600
10	RFu#6/AP1	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL578150.1	Completely depressed	~700
11	RFu#6/AP1	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL564689.1	Completely depressed	~650
12	RFu#6/AP3	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL578150.1	Completely depressed	~700
13	RFu#6/AP3	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL571997.1	Completely depressed	~660
14	RFu#6/AP5	Non-normalized library—*P. polycephalum* cDNA	gb|EC756915.1	Completely depressed	~700
15	RFu#6/AP5	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL578150.1	Completely depressed	~670
16	RFu#6/AP5	*P. polycephalum* starvation stress library *P. polycephalum* cDNA	gb|EL571997.1	Completely depressed	~450

Zerumbone treatment during S phase resulted in maximum DNA synthesis inhibition with respect to control as revealed by FACS analysis without being genotoxic as no comets could be observed. Reports directly linking zerumbone and DNA synthesis inhibition were not encountered barring a single report on HT-29 human colon cancer cells based on FACS analysis (Kirana et al. 2003). Incidentally, replication of ribosomal DNA genes of *Physarum*, which exist as palindromic extrachromosomal elements, is confined to the last two thirds of S phase and all of G2 phase (Hardman 1986). Interestingly, the DNA synthesis inhibitory action of zerumbone was also evident in G2 phase, although expectedly to a lesser extent. This could be likely a consequence of rDNA synthesis inhibition by zerumbone treatment. Taken together, the unique situation in *Physarum* with DNA synthesis also occurring in G2 lends credence to the inhibitory effect of zerumbone on DNA synthesis per se even outside S phase.

Transcript profiling by differential display is a popular method to discover differences in gene expression. The effect of zerumbone studied at the transcription level during the cell cycle phase in which the compound elicited maximal differential mitotic delays—early G2—showed distinct variations in comparison to controls. In addition to the three newly induced and 15 completely suppressed amplicons, 38 were found to be downregulated while 31 were upregulated. Of the selected clones sequenced, a little less than 50 % matched with stress-related transcripts from *Physarum*, 25 % were found to be *Physarum* transcripts of unknown function, and the rest were found to be hitherto unreported. This also brings to focus the link between the cellular networks involving stress-related gene expression and cell cycle regulation, namely G2 arrest induced by zerumbone in *Physarum.*

In conclusion, the present study shows that zerumbone elicits G2 arrest in a concentration and cell cycle phase dependant manner on *Physarum* with early G2 being the most sensitive phase. Protein profiles indicated that zerumbone apparently inhibited cyclin B1 expression leading to G2 arrest. The results also showed that zerumbone inhibited DNA synthesis both in S phase as well as in G2 into which rDNA synthesis continues to occur in *Physarum* cell cycle; genotoxicity was also not observed at the concentrations tested. Zerumbone treatment resulted in variations with respect to specific transcripts including many which were found to be stress related suggesting that these may have an involvement in cell cycle-related phenomenon such as G2 arrest.

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Conflict of interest The authors declare no conflict of interest.

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