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Addition of citral controls ROS and reduces toxicity in 5-fluorouracil treated *Schizosaccharomyces pombe* cells

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In systemic therapy, chemotherapeutic drugs, often, cause considerable side effects; and combination of natural compounds lessen the extent of such effects. In the present study, combined effect of citral and 5-fluorouracil was studied in *Schizosaccharomyces pombe* cells. The antagonistic combination index found was at 0.01 and 0.025 mM of citral with 40 µg or higher concentration of 5-fluorouracil. The combined treatment was so effective that higher number of cells underwent apoptosis compared to individual treatment of 5-fluorouracil. Citral controlled ROS levels and increased survival of normal cells. Several differentially expressed proteins observed in the citral treatment could further help understanding its mechanism of action.

Keywords: Apoptosis, Chemotherapy, Lemongrass, Phytomedicine

Despite the advancement in pharmaceutical research, management of side effects caused by chemotherapeutic drugs remains a major challenge. Use of natural compounds along with chemotherapeutic drugs is an emerging idea for managing such side effects. In the present study, we used 5-Fluorouracil (5-FU), a widely used chemotherapy drug like cyclophosphamide, methotrexate, etc., along with citral, a phytomedicine, to find out the synergy between them, and to study the effect of toxicity on *Schizosaccharomyces pombe* (*S. pombe*) cells.

S. pombe, is a model organism to study the cellular mechanism in medicinal[1] as well as apoptotic research[2,3]. It is convenient to establish basic principles of synergy in *S. pombe*. Citral (3,7-dimethyl-2,6-octadienal), a key component of essential oils, is a mixture of two isomers, geranial and neral, which are acyclic α, β-unsaturated monoterpene aldehydes naturally occurring in many essential oils. Lemon grass (*Cymbopogan citrates*) contains 65-85 % citral[4]. Citral has been shown to exhibit antimicrobial, antifungal, antioxidant and free radical scavenging activities in mice[5]. It is devoid of major toxicity and has demonstrated carcinogenic potential in both mice and rat[6,7]. Further, citral exerts antimutagenic effect against cyclophosphamide induced mutagenicity[8]. Also, it induces caspase-3 mediated apoptosis of leukemia cells without affecting normal spleen cells and thymocytes[9,10]. Although assessed as independent chemoprevention agent, use of citral in combinational therapy is not studied evidently. Being a major constituent of lemongrass, which is widely used as drinking tea nowadays, study of citral in combination with 5-FU may reveal more insight.

Materials and Methods

Citral, 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA), 5-Flurouracil, acridine orange and ethidium bromide were purchased from Sigma-Aldrich. The components of culture media were purchased from Hi-media.

Yeast strain and Growth conditions—*Schizosaccharomyces pombe* wild type strain was grown in liquid YES medium on a rotary shaker at 150 rpm at 30 °C. Cell growth was measured by taking OD at 595 mM as well as counting cells using haemocytometer.

Effect of Citral and 5-flurouracil on S. pombe cell growth and cell viability—Citral concentration ranging from 0.01-2.0 mM and 5-FU at a concentration ranging from 0-500 µg/ml were used to treat the *S. pombe* cells. Equal numbers of *S. pombe* cells were inoculated in liquid YES medium and allowed to grow up to exponential phase. Various

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concentrations of citral and 5-FU were added to exponentially growing cells independently and in various combinations. The cells were further allowed to grow for 24 h and growth rate was determined by cell counts using haemocytometer. Cell viability was assessed by trypan blue viability assay.

Drug response curve and calculation of combinational index—Exponentially grown cells were treated with various concentrations of 5-FU and citral for 24 h. After measuring % viability, drug response curve for citral and 5-FU was generated and IC_{50} was obtained. The combination effect was expressed as combination index (CI) calculated using formula: CI= (D1/Dx1) + (D2/Dx2) where, D1 and D2 are the concentration of each agent used in combination; Dx1, Dx2 are the concentration of single compound producing the same effect as combination treatment[11]. The CI values were defined either as synergism (CI < 1), additive effect (CI = 1) or antagonism (CI > 1).

Measurement of ROS generation in citral and 5-FU treated cells—Intracellular production of ROS in citral and 5-FU treated and control *S. pombe* cells was assessed with the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA). The DCFHDA (10 µM) was added to the exponentially growing *S. Pombe* cells and cells were allowed to grow further for 30 min. This was followed by addition of citral and 5-FU at defined concentration and further incubated for 3 h. Fluorescence was read at 485 nm excitation and 535 nm emission in a fluorescence plate reader (Hitachi 7000, Japan). The wells containing drug and/or citral but not DCFHDA acted as blanks for each sample. ROS production was expressed as % increase in fluorescence relative to untreated control cells.

Ethidium bromide/Acridine Orange staining—The staining solution was prepared by making 0.3 ml of 10 mg/ml acridine orange (AO) and 1.0 ml of 10 mg/ml ethidium bromide (EB) in 95% ethanol up to 100 ml with PBS. The exponentially growing *S. pombe* cells were treated with citral and 5-FU in combination or independently for one generation and were harvested. Treated and untreated cells were washed with PBS. A 20 µl of dye mixture (10 µl AO and 10 µl EB) was mixed with 100 µl of cell suspension in PBS. After incubation for 5 min at room temperature, 10 µl of the dye-cell mixture was placed on a clean microscope slide and then covered with a 22-mm coverslip. Cells were examined under fluorescence microscope at 40X. After counting 300 cells, the numbers of each of four cellular states were scored: (a) viable cells with normal nuclei (V, bright green chromatin with organized structure); (b) early apoptotic cells (EA; bright green chromatin highly condensed or fragmented); (c) late apoptotic cells (LA; bright orange chromatin highly condensed or fragmented); and (d) necrotic cells (N, bright orange chromatin with organized structure).

Protein extraction and estimation—*S. pombe* cells, with or without treatment of citral and 5-FU were harvested and subjected to cell lyses for protein extraction. Cells washed with distilled water and to the palette, glass beads and extraction buffer (50 mM Tris (pH=7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1mM PMSF) were added. The mixture was vortexed for 10 min while keeping on ice in between followed by centrifuging at 10000 rpm at 4 °C for 10 min, and the supernatant was collected. Protein was estimated by Folin-Lowry method[12].

SDS-Polyacrylamide gel electrophoresis—SDS-PAGE was carried out using 12% resolving gel and 7.5% stacking gel. After electrophoresis, the gel was stained with 0.1% coomassie brilliant blue (CBB) R-250 dye prepared in a mixture of methanol, acetic acid and distilled water at ratio 40:10:50, respectively. Image of stained SDS-PAGE gel was captured and analyzed by the image analyzer (Alpha Digi Doc, USA).

Statistical analysis—All experiments were repeated at least three times. The results of multiple experiments are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's high significant difference (HSD) test. SPSS software 17 was used for the statistical analysis.

Results

Effect of Citral on growth of S. pombe cells—Fig. 1A depicts the result of treatment of wild type *S. pombe* cells with citral concentrations (0.01-1.6 mM) for 7, 14 and 28 h. Decreased cell growth with increasing concentration of citral was observed. Although, at lower concentrations of citral (0.01 and 0.025 mM) the % viability was almost equal to control cells, significant reduction in *S. pombe* cell number was observed at and above 0.05 mM concentration. The IC_{50} obtained from drug response curve was 0.1 mM (Fig. 1B).

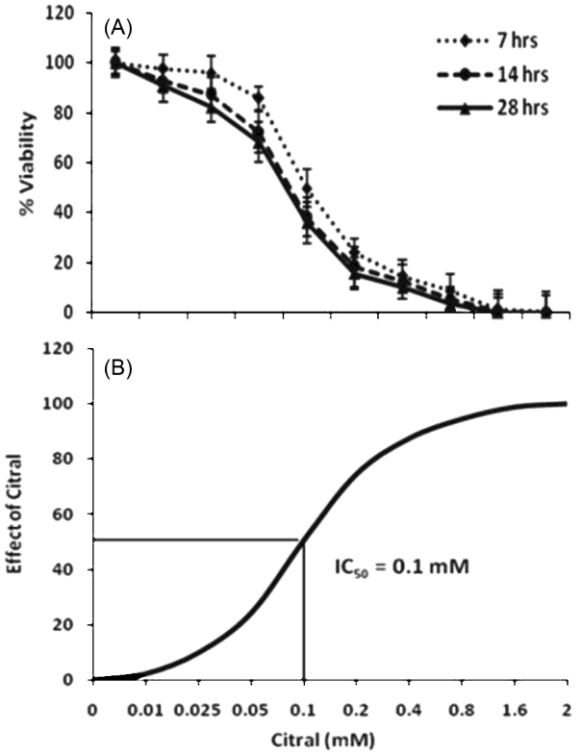


Fig.1—(A) Effect of different concentration of citral on growth of *S. pombe* cells at 7, 14 and 28 h. Growth rate was determined by cell counts using haemocytometer and cell viability was assessed by trypan blue viability assay. Data are presented as Mean ± SD. (B) drug response curve of citral and IC_{50} measurement. Effect of citral represents the survival of cells after citral treatment.

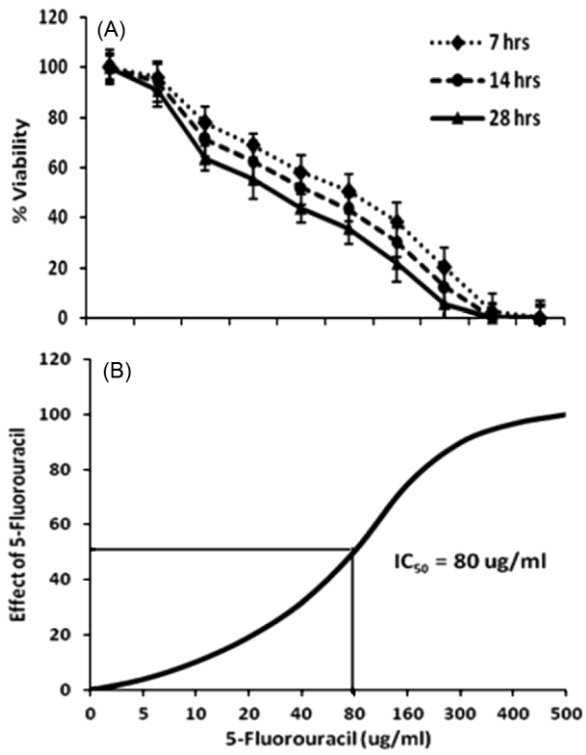


Fig. 2—(A) Effect of different concentration of 5-FU on growth of *S. pombe* cells at 7, 14 and 28 h. Growth rate was determined by cell counts using haemocytometer and cell viability was assessed by trypan blue viability assay. Data are presented as Mean ± SD. (B) drug response curve of citral and IC_{50} measurement. Effect of 5-FU represents the survival of cells after 5-FU treatment

Effect of 5-Fluorouracil (*5-FU*) *on growth of S. pombe cells*—The effect of chemotherapy drug 5-Fu on *S. pombe* cells was studied by treating the cells with variable concentration of 5-FU (0-500 µg/ml) for different time period (7, 14 and 28 h). A dose dependent decrease in cell growth was observed after treatment with 5-FU. However, a significant decrease in cell growth was observed at concentrations ranging from 20-500 µg/ml (Fig. 2A). The IC_{50} obtained from drug response curve was 80 µg/ml (Fig. 2B).

Effect of citral and 5-FU on growth of S. pombe cells—To assess the effect of citral on 5-FU treated *S. pombe* cells, they were treated with various concentration based combinations of citral and 5-FU for 24 h. A deviation in growth rate was observed upon treatment of citral. As apparent in Fig. 3, when treated with a combination of increasing concentration of citral and 5-FU, the *S. pombe* cells demonstrated significant cell survival effect compared to sole treatment of 5-FU (P <0.05). Out of six different combinations tried, the antagonistic effect of citral with 5-FU was observed at

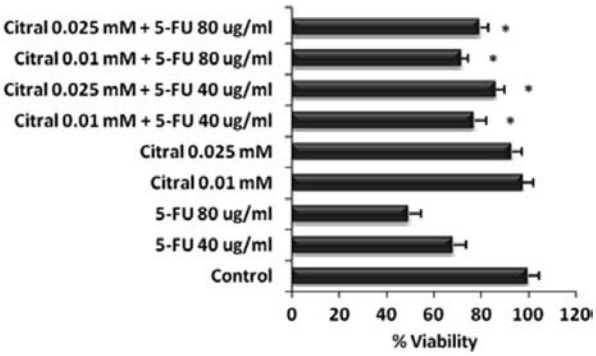
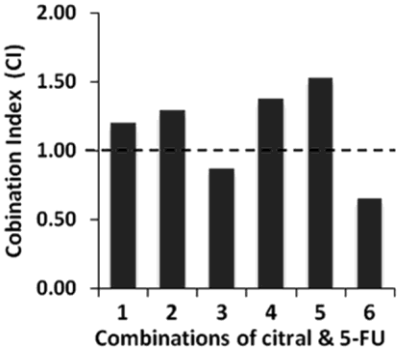


Fig. 3—Effect of citral and 5-FU, independent and in combination after 24 h on % viability of *S. pombe* cells. *S. pombe* cells were treated with 0.01 and 0.025 mM citral concentration and/or 5-FU at a concentration of 40 and 80 µg/ml. Growth rate was determined by cell counts using haemocytometer and cell viability was assessed by trypan blue viability assay. Data are presented as mean ± SD.*P <0.05.

0.01 and 0.025 mM citral and 40 and 80 µg/ml 5-FU (CI value >1). A higher concentration of citral (0.05 mM) with 40 and 80 µg/ml of 5-FU showed the CI value less

than 1 suggested synergism (Fig. 4). In the present study, antagonistic concentration of citral and 5-FU was used for further studies.

Quantification of Apoptosis by AO/EB staining—Whether the decrease in growth of *S. pombe* cells treated with citral and 5-FU effect was due to induction of apoptosis or not was assessed by AO/EB staining of cells. The staining of treated and untreated *S. pombe* cells by AO/EB had facilitated the microscopic identification of apoptotic cells and distinguishing of apoptotic cells from necrotic. As shown in Fig. 5A , viable cells had uniform bright green nuclei with an organized structure; (A') early apoptotic cells had green nuclei with intact membrane but cleaved DNA; (A) late apoptotic cells had orange to red nuclei with condensed or fragmented chromatin and necrotic cells had uniformly orange to red nuclei with a condensed structure. Percent distribution of



Combinations of citral & 5-FU	Citral (mM)	5-FU (µg/ml)
1	0.010	40
2	0.025	40
3	0.050	40
4	0.010	80
5	0.025	80
6	0.050	80

Fig. 4—Effect of various concentrations of 5-FU + citral on combination index of affected *S. pombe* cells. A CI value significantly less than 1 indicated synergism, a CI not significantly different from 1 indicated addition, and a CI significantly higher than 1 indicated antagonism.

apoptotic *S. pombe* cells based on AO/EB stained nuclei is shown in Fig. 5B. The 5-FU treated cells showed necrotic cell death. Control and citral treated cells had significantly high number of viable cells compared to 5-FU treated cells. Citral induced apoptosis and increased the survival of cells treated with 5-FU. Even the combined treatment of citral and 5-FU induced the apoptotic cell death.

ROS generation in S. pombe cells treated with citral and 5-FU—Since many genes are known to encode proteins that generate ROS in cells undergoing apoptosis[13], we assessed ROS generation in *S. pombe* cells treated with citral and 5-FU using DCHFDA. Compared to control cells, the cells treated with 5-FU showed significantly higher % of ROS (P <0.05). While nearly similar % of ROS values were found with lower concentration of citral treatment. A decline in % ROS was observed in the cells treated with increasing concentration of citral. As shown in Fig. 6, combined treatment of 5-FU and citral significantly reduced the % ROS compared to sole treatment of 5-FU (P <0.05).

Effect of citral and 5-FU on Protein profile—To have an insight into changes in the protein profile due to citral and 5-FU treatment in *S. pombe* cells, protein extraction and quantification from citral and 5-FU treated (either alone or in combination) and untreated cells were carried out. The results have shown that individual treatment of 5-FU and citral resulted in increased protein levels compared to control (P <0.05). A combined treatment of citral and 5-FU also increased the protein levels compared to control (Fig. 7A). These differences in protein yield were reflected in the

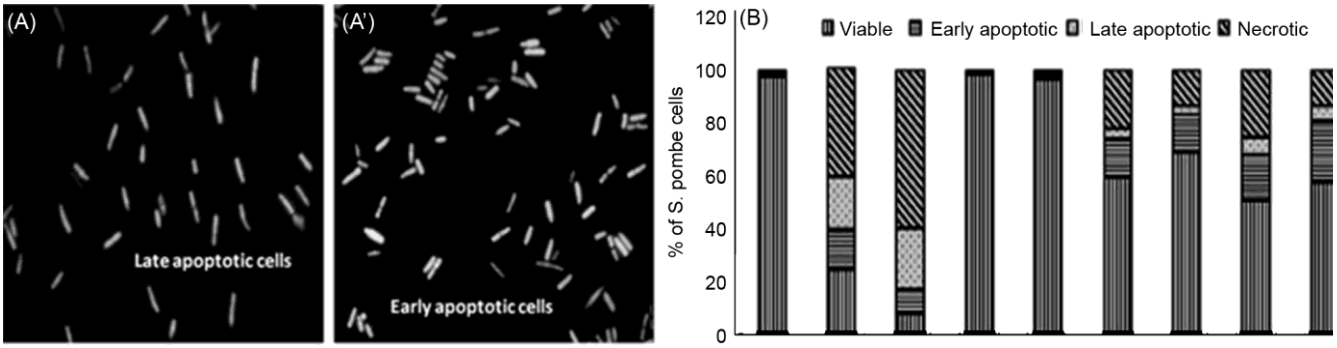


Fig. 5—(A) Citral and 5-FU treated (either independently or in combination) *S. pombe* cells were stained with dual stain AO/EB and observed under fluorescence microscope 40x. Treatment of cells with 5-FU (80µg/ml) shows late apoptotic cells (A) whereas combined treatment of 5-FU (80µg/ml) and citral (0.025 mM) shows early apoptotic cells (A'). (B) Cells are expressed as % apoptotic cells of the total cells counted in different fields taken as 100%. Bar 1 - represents control, bar 2-cells treated with 5-FU 40 µg/ml, bar 3-cells treated with 5-FU 80 µg/ml, bar 4-cells treated with citral 0.01 mM, bar 5-cells treated with citral 0.25 mM, bar 6-cells treated with citral 0.01 mM + 5-FU 40 µg/ml, bar 7-cells treated with citral 0.01 mM + 5-FU 80 µg/ml, bar 8-cells treated with citral 0.24 mM + 5-FU 40 µg/ml and bar 9 - cells treated with citral 0.25 mM + 5-FU 80 µg/ml.

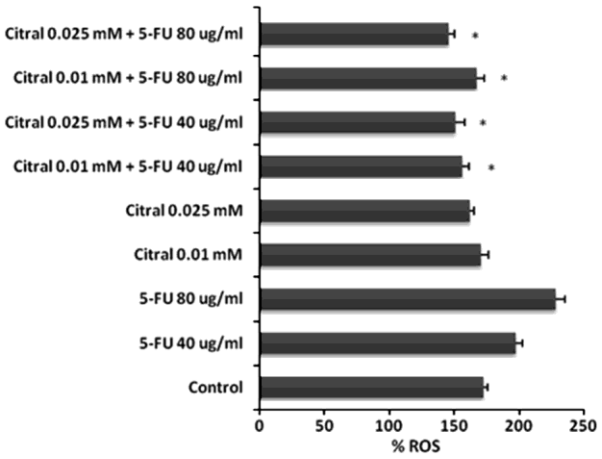


Fig. 6—Percentage of ROS generation in citral and 5-FU treated, either in combination or independently and untreated *S. pombe* cells. Cells incubated with DCHFDA for 30 min followed by treatment with drug and citral and further incubated for 3 h. Fluorescence was read at 485 nm excitation and 535 nm emission and ROS production was expressed as % increase in fluorescence relative to untreated control cells. Data are presented as mean ± SD.

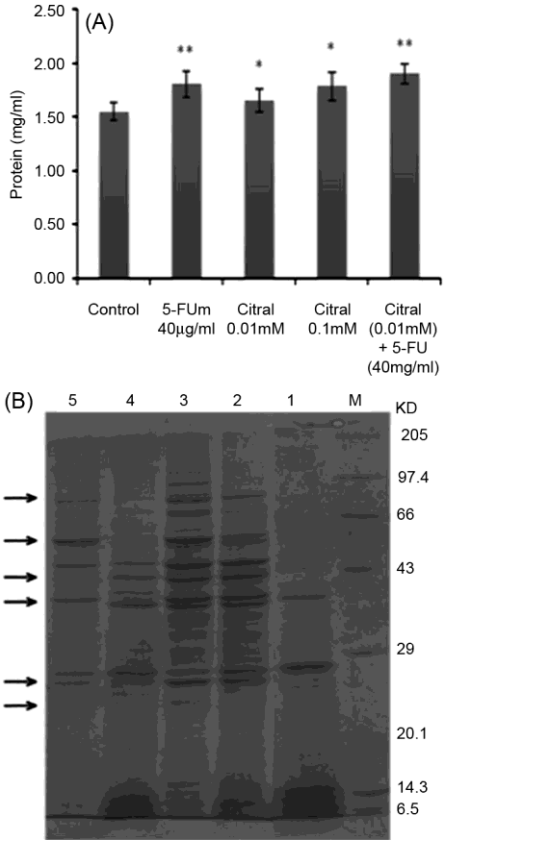


Fig. 7—(A) Effect of citral, 5-FU and their combination on cellular protein levels. Data represents the mean ± SD of one of the three similar experiments each performed in triplicate. **P* <0.05, ***P* <0.01. Bb) Equimolar total extracted proteins were run in SDS PAGE. Gel showed the expression of new proteins as well as change in the level of expressed proteins when compared with control bands' pattern (Shown as arrows on left side). M, Marker; Lane 1, Control (untreated cells); Lane 2, Drug (5-flurouracil 40µg/ml); Lane 3, Drug + citral (40µg/ml + 0.025 mM; Lane 4, citral (0.01 mM); Lane 5, citral (0.1 mM).

results of SDS-PAGE (Fig. 7B), confirming the differential expression of proteins in cells treated with 5-FU and citral either in combination or independently at variable concentrations. Gel showed the altered expression of proteins as well as changes in the level of expressed proteins compared to control bands' pattern. The R*f* of different new and highly expressed proteins' bands were calculated, from which the probable molecular size of these proteins were calculated. The identified molecular sizes of differentially expressed proteins were 84, 57.63, 39, 29.14 and 24.75 KD.

Discussion

Citral exhibited time and dose dependent effect on *S. pombe* cells. At lower concentration, citral did not show a growth inhibitory effect on *S. pombe* cells. However, treating the cells with increasing citral concentration decreased the cell viability. The treatment of *S. pombe* cells with various concentrations of 5-FU significantly decreased the cell growth.

In the present study, the IC_{50} of 5-FU obtained for *S. pombe* cells was 80 µg/ml. Similar study on *S. cerevisiae* reported 110 ± 20 µM[14]. The addition of citral to the 5-FU treated *S. pombe* cells significantly increased the survival of cells. While trying various combinations of citral and 5-FU, combined treatment of 0.01 and 0.025 mM of citral with 40 and 80 µg/ml of 5-FU showed significant increase in the *S. pombe* cells survival. CI obtained at this concentration revealed that the antagonist effect was supportive of the use of citral to protect the toxicity caused by 5-FU on normal *S. pombe* cells.

While analyzing the cellular death, it was observed that the 5-FU treated *S. pombe* cells underwent necrotic cell death. Along with increasing cell survival, citral addition to 5-FU treated cells shifted this necrotic cell death to apoptotic. Citral was also found to induce apoptosis in a time and dose dependent manner in NH4 cells[10] and in MCF 7 cells[15].

ROS generation is common observations in almost all cells undergoing apoptosis[13]. Citral treatment reduced the generation of ROS compared to control cells. This observation explained the dose dependent antioxidant activity of citral on normal cells.

Moreover, the combined treatment of citral and 5-FU further decreased the level of % ROS in *S. pombe* cells, presenting the defensive antagonistic effect of citral with 5-FU.

The analysis of protein expression demonstrated the alteration in expression of induced proteins. The induced proteins might be of glycoprotein group (84KD), heat shock proteins (20-28 KD) and ribonucleosome proteins (55-60 KD), as compared with the molecular weight from the literature. However, further confirmatory experiments would reveal the mechanism of citral.

In summary, the addition of citral increased the survival of *S. pombe* cells treated with 5-FU. As a consequence, in apoptotic event, the 5-FU treated cells were shifted from late apoptosis and necrosis to early apoptosis upon treatment with citral. Induction of apoptosis was resulted as a generation of ROS. Study of induced protein will be able to reveal the mechanisms of apoptosis carried out by citral.

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