INTERNATIONAL JOURNAL OF APPLIED AND EXPERIMENTAL BIOLOGY
(ISSN PRINT: 2790-6523; ISSN ONLINE: 2790-6531)
2023, Vol. 2, NO. 2, 89-96.
doi: 10.56612/ijaeb.v1i1.57
https://eminentscientists.com/

Apoptotic potential of geranyl acetate in HepG2 liver cancer cells

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Abstract
Natural compounds having apoptotic effects are a new potential source for anti-cancer drugs. Numerous plant phytochemicals have proven anti-cancer properties by inducing programmed cell death (apoptosis). The disease known as cancer is characterized by the uncontrollable growth of some body cells and their spread to other body regions. Worldwide, cancer is a major cause of death. The objective of the current study was to assess apoptotic potential of geranyl acetate on HepG2 cancer cell line. Cell death and cell viability in groups were assessed using the MTT, trypan blue and crystal violet to assess anti-proliferative effect. P53 ELISA was carried out to assess apoptosis. When HepG2 cells were exposed to geranyl acetate they exhibited increased cytotoxicity, decreased viability, proliferation, and increased apoptosis. It is concluded that geranyl acetate has the potential to cause apoptosis via P53 in HepG2 cells. It can also prevent cancer cell growth and proliferation.

SECTION
Human Biology (HB)

HANDLING EDITOR
Ashraf, M.A.B. (HB)

ARTICLE HISTORY
Received: 13 Jan, 2023
Accepted: 14 Apr, 2023
Published: 3 May, 2023

KEYWORDS
Apoptosis;
Programmed cell death;
HepG2 cancer cell line;
Cancer cell growth;
Liver cancer cells

Introduction

At global level, cancer is regarded as fatal disease (Rawla et al., 2019). Cancer is considered as 4th cause of death all over the world (Sayiner et al., 2019). Cancer has become a rapid developing disease in the world as many casualties occur every year (Labgaa et al., 2019). Men are more prone to the disease as compared to women. Even in some countries, the risk of cancer has reached at an alarming stage, because young people less than 20 years of age were affected by cancer, whereas in some other countries, cancer has not spread much as some of more than 50 years of age people were affected by cancer, but lower age people were not affected (Bosch et al., 2004). The main risk factors in case of cancer are the causes of such variations in different regions of the world. As there are multiple reasons of cancer, so some areas have common risk factors such as hepatitis infections which are caused by fungi in contaminated food. International agencies have also classified these infections as carcinogenic to humans, such as hepatocellular carcinoma. Other factors include diabetes, obesity and alcohol intake excessively (Thorgeirsson and Grisham, 2002). Sometimes, liver cancer is associated with liver complications such as cirrhosis and fibrosis. Hence, history of liver disease can determine prognosis of the disease (Böttcher and Pinzani, 2017) in which certain changes at cellular level are responsible for cancer proliferation such as injury to liver cells resulting in production of oxidative stress markers (Rolo et al., 2012).

Apoptosis is cell death due to programmed mechanism installed in cells by nature. These damaged cells were studied and it was found that cell death occurred due to damage of DNA of cells during mitosis

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TO CITE THIS ARTICLE: Ahmad, I., Maqbool, T., Naz, S., Inamullah, Hadi, F., Atif, M. (2023). Apoptotic potential of geranyl acetate in HepG2 liver cancer cells. *International Journal of Applied and Experimental Biology* 2(2): 89-96.

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(Pistritto et al., 2016). During apoptotic process, cellular machinery is involved, which is a complex mechanism, and hence, difficult to understand. There are multiple cellular signals involved, which make this apoptosis possible at cellular level. Certain cellular mechanisms can be disturbed to induce apoptosis in cells such as caspase-mediated pathways. These pathways are of two types, i.e., intrinsic and extrinsic (Dong et al., 2019). As a result of these pathways, certain alterations occur in the cells at molecular level, leading to apoptosis (Bedoui et al., 2020). Some mechanisms of cells involve special proteins which promote apoptosis, called as pro-apoptotic proteins; some are inhibitory to apoptosis and they are regarded as anti-apoptotic proteins (Montero and Antony, 2018). Balance between both types of proteins determine the condition of cells to induce apoptosis or not. Hence, the mechanism to induce apoptosis in cancer cells can prevent body from tumor disease in long-term (Pylayeva et al., 2011). Otherwise, if cancer cells succeed to prevent from apoptosis in body, then they proliferate and cause malignancy and resistance to therapies (Evan and Vousden, 2001). For this reason, anti-apoptosis is considered a hallmark of cancer (Greenhough et al., 2009). Therefore, therapies involving targeting those molecules which are causes of cancer treatment resistance, are the key to success (Min and Lee, 2022).

Monoterpenes are compounds that have strong physiological effects and high taste and smell, and are often used as flavor in food business, pharmaceutical industry, agrochemical industry, and cosmetic industry (Klimek et al., 2020). Monoterpenes are used to make scents and perfumes and are mostly produced by plants (Mahmoud and Croteau, 2002). Moreover, these compounds displayed different therapeutic properties such as anticancer, hypolipidemic, fungicidal, bactericidal, anti-oxidant, anti-inflammatory, analgesic and sedative activities (Rodenak et al., 2020). Geranyl acetate is one of the potential monoterpenes known in nature (Wu et al., 2018). Like other compatible monoterpenes it has a multitude of effects on human metabolism perturbed under the impact of certain diseases including cancer (Qi et al., 2018). Thus, in the present study, we evaluated the anticancer potential of geranyl acetate, a monoterpene, through its apoptotic and anti-proliferative potential in the liver cancer cells HepG2.

Materials and Methods

Sampling of HepG2 cell line

Liver cancer (HepG2) cell line was provided by cell culture facility established at The University of Lahore. Liquid nitrogen cylinders were used to store the cryovials, which were thawed before use. After thawing, the cells were cultured and nourished by a medium called as Dulbecco's Modified Eagle Medium (DMEM). The geranyl acetate was purchased from Thermo (Catalog number A19864.14), which was further diluted in the DMEM without FBS.

Culturing of HepG2 cell-line

The cells were placed and cultured in T75 culture flasks containing a medium as DMEM, and heat-inactivated fetal bovine serum was added to the medium, along with addition of antibiotics, i.e., streptomycin and penicillin. When the cells were 75-85% confluent, they were sub-cultured by using trypsin-EDTA. Cellular suspension obtained after trypsinization was added to a 15 mL falcon tube and then was centrifuged at 1450 RCF for five min. The remaining supernatant was discarded and the pellet was re-suspended in the medium (Abid et al., 2020).

Treatment of HepG2 cell line followed by geranyl acetate

A 96-well plate was used to cultivate the HepG2 cell line for cell proliferation and biochemical tests. The HepG2 cells were divided into five groups. Four of the groups received geranyl acetate treatment as 50, 100, 200 and 400 μM, while one group served as the control. For evaluation of apoptotic activity, the cells were treated for 24 h. After analysis, a maximum higher dose was chosen for further experimentation. Plates with 96-wells were used for MTT, crystal violet, trypan blue, scratch and Elisa assays, and a 6-well plate was used for the morphological analysis of HepG2.

Cell viability assays (MTT assay)

After 24 h of treatment with geranyl acetate the treatment media was removed and 100 μL of DMEM added along with 25 μL of MTT solution. Formazan crystals were dissolved in DMSO (Invitrogen Inc., USA). Finally, the absorbance was measured at 570 nm (Maqbool et al., 2019).

Crystal violet assay

The crystal violet staining was used to measure cell viability as well. After 24 h of treatment, the treatment media was removed, the plate was washed and incubated with 0.1% crystal violet dye in 2% ethanol for 15 min at room temperature. Wells were thoroughly cleaned and the dye was disposed of

carefully to prevent the cells from lifting out of the wells. The stain was then solubilized by adding 100 µL of 1% SDS to each well and allowed for 5 to 10 minutes. Ultimately, an absorbance measurement at 595 nm was made on a microtiter plate reader (Nasir et al., 2017)

Trypan blue assay

Trypan blue was used to count dead cells when the treatment was administered. Washing of the cells was performed with PBS and incubated in trypan blue dye (Invitrogen Inc., USA) for 15 minutes. After that, the cells were again washed with the same saline and examined under a microscope. Dead cells were stained in blue (Hadi et al., 2020)

Enzyme-linked Immunosorbent Assay (ELISA)

The secretome obtained from the treated cells was collected and anti-p53 ELISA (bioassay technology, shanghai) was performed. All chemicals, i.e., samples, standard solutions and reagents, were prepared as directed. Before use, all reagents were taken out from the freezer and thawed at room temperature. An aliquot of 10 µL anti-p53 antibody, 50 µL streptavidin-HRP and 40 µL of the sample were added and incubated for 60 mins at 37 ^{o}C. Then the plate was washed for five times. After that 50 µL of the substrate solution A and 50 µL of the substrate solution B was added to each well. Again, the plate was incubated for 10 min at 37 ^{o}C. Then 50 µL stop solution was added to each well and OD was measured at 450 nm within 10 minutes.

Statistical analysis

GraphPad Prism 5 was used for statistical analysis. One-way ANOVA was used to examine variance in all experimental group data that were expressed in triplicate as mean $\pm$ SD. The t-test was worked out using the GraphPad Prism 5 to ascertain if the data of any of two groups are significantly different from each other.

Results

Morphological analysis of untreated and treated cells with geranyl acetate

The treated HepG2 cells with different concentrations of geranyl acetate 50 µM, 100 µM, 200 µM, and 400 µM showed large numbers of dead cells in a dose-dependent manner. Morphological traits were observed by a Floid Cell Imaging station.

MTT (Proliferation Assay)

Cytotoxicity of geranyl acetate was observed through the MTT assay, which is a reliable method for measuring cell viability. The results showed a significantly less viable cells in the treatment groups compared to those in the control group in a dose-dependent manner as presented in Table 1 and Figure 2.

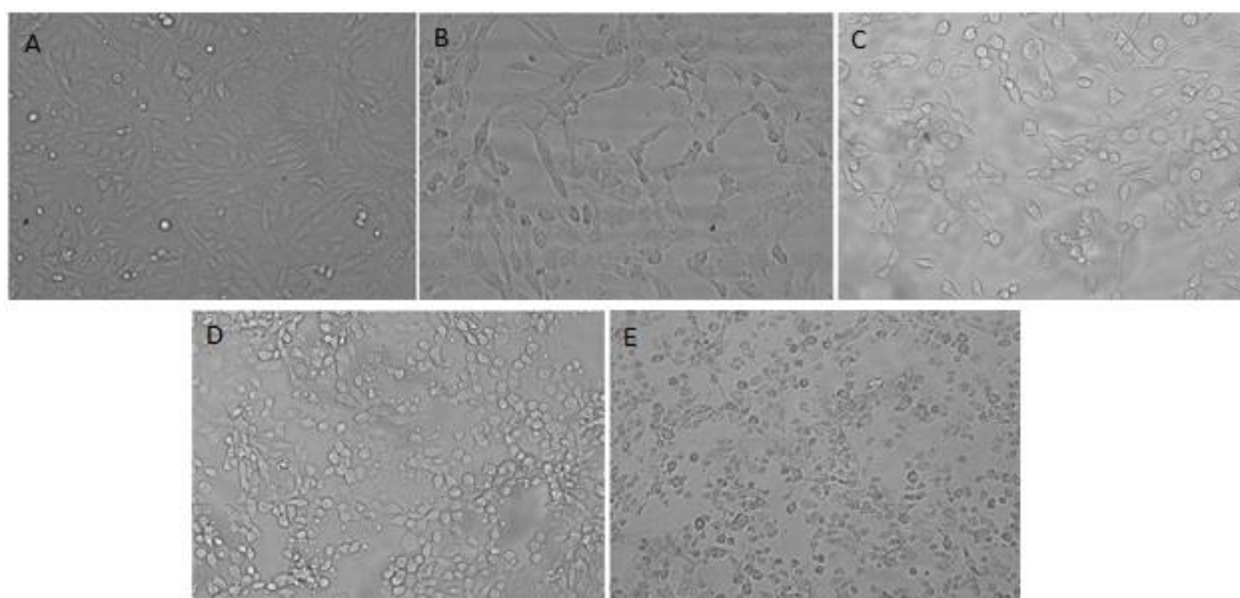

Figure 1. Morphology of treated HepG2 cell line: (A) Untreated (B) 50 µM geranyl acetate, (C)100 µM geranyl acetate, (D) 200 µM geranyl acetate, and (E) 400 µM geranyl acetate

Crystal violet (Living Cells Detection)

Additionally, the crystal violet staining was used to determine the vitality of the cell line. As shown in Figure 3 and Table 2, the results for the geranyl acetate treatment reduced the number of live cells in HepG2 cells as compared to that in the untreated cells.

Trypan blue (Cell viability detection)

Trypan blue was used to count dead cells to determine whether geranyl acetate might have anti-apoptotic properties. The HepG2 cells treated with geranyl acetate showed a considerably higher number of blue-colored cells, suggesting more dead cells in comparison with the untreated liver cancer cells group.

Table 1. The Proliferation assay shows the mean values and standard deviations of different groups

MTT Assay	Untreated	Treated with varying levels of geranyl acetate			
		50 µM	100 µM	200 µM	400 µM
Mean ± SD	0.449 ± 0.06	0.330 ± 0.017	0.332 ± 0.044	0.295 ± 0.007	0.104 ± 0.003

Table 2. The crystal violet staining shows mean values and standard deviations of Untreated and treated with geranyl acetate

Crystal Violet	Untreated	Geranyl acetate 400 µM
Mean ± SD	0.868 ± 0.00196	0.046 ± 0.00342

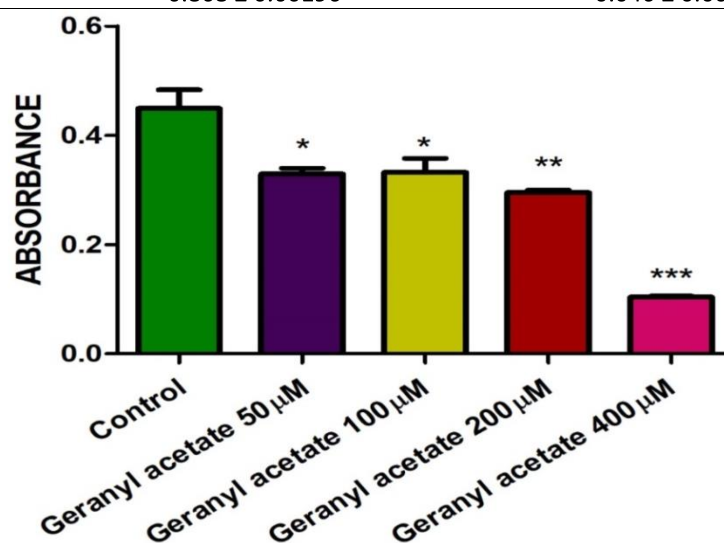

Figure 2. Geranyl acetate-induced cytotoxicity in HepG2 cells using the MTT test. The bar graph demonstrates the significant difference between the control and the treatment. Values were computed as the mean ± the standard error. *, **, * significant at 0.05, 0.01, and 0.001 levels, respectively**

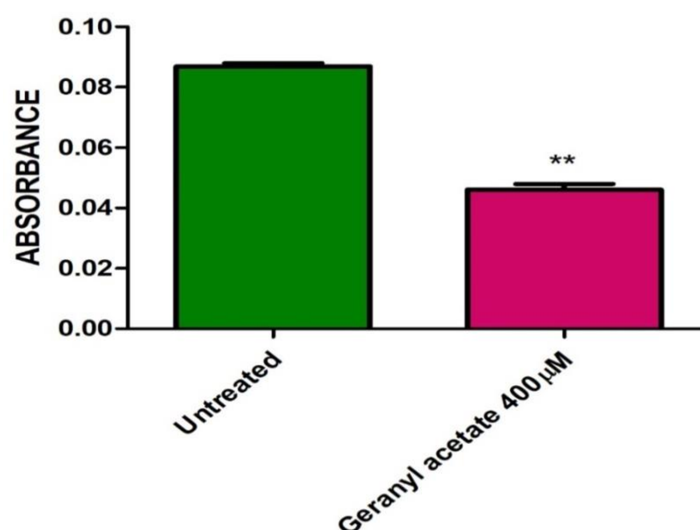

Figure 3. Crystal violet analysis of living cells in geranyl acetate-treated HepG2 cells and untreated HepG2 cells. According to bar graphs, geranyl acetate-treated HepG2 cells had fewer live cells than untreated HepG2 cells. Values were computed as the mean minus the standard deviation of the mean. ** *P* < 0.01.

Table 4. The trypan blue detection method shows mean values of the control group and the geranyl acetate group

Trypan Blue	Control	Geranyl acetate 400 µM
Means	15.0 ± 2.00	30.0 ± 4.36

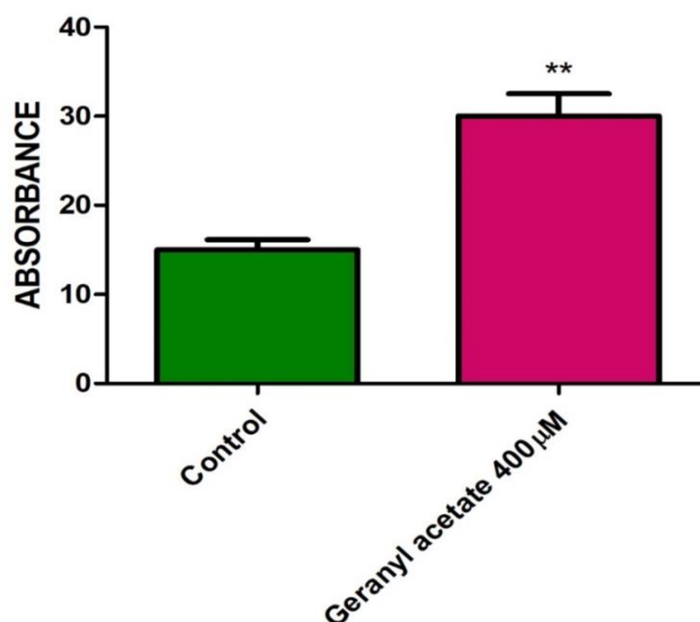

Figure 4. Analysis of dead cells count in untreated vs treated cells with geranyl acetate. The bar graph demonstrates that the proportion of dead cells was higher in the geranyl acetate-treated samples than that in the untreated ones. ** *P* < 0.01

ELISA - *Increased level of apoptosis in post-treated HepG2 cells via P53 ELISA*

ELISA of p53 showed higher level of apoptotic cells in the treated group compared to that in the untreated (Figure 6 and Table 5). Since p53 is a crucial marker of apoptosis, its level was raised in the cell lines that had been treated with geranyl acetate.

Table 5. Level of apoptosis of P53 via ELISA

ELISA p53	Untreated	Geranyl acetate 400 µM
Mean ± SD	0.543 ± 0.0404	0.981 ± 0.0265

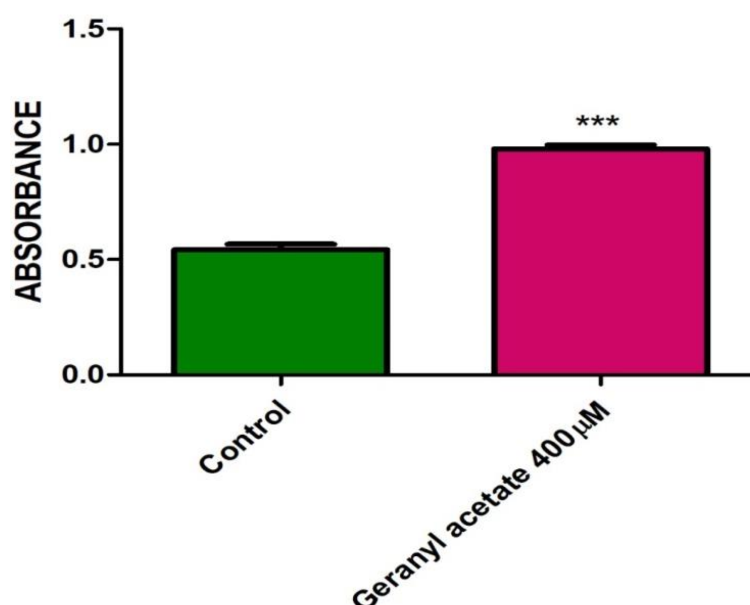

Figure 5. Level of apoptosis in the treated vs untreated HepG2 cells. This bar graph illustrates a significantly greater level of p53 in geranyl acetate-treated HepG2 cells as compared to that in the untreated. * shows a significant difference between the untreated and treated group at 0.001 level of probability.**

Discussion

Cancer is one of the leading causes of mortality worldwide (Bray et al., 2021). The majority of cancer-related deaths result from lung, liver, stomach, colorectal, breast, prostate, and esophageal cancers (Cao et al., 2021). Cancer is predicted to rise by 70% over the next 20 years (Asrani et al., 2019). Chemotherapy drugs affect the stage by a variety of ways, including promotion of tumor cell differentiation, acceleration of cell death, and suppression of cellular proliferation (Ouyang et al., 2012). Many naturally occurring compounds have been employed for specialized medicinal objectives, but it is evident that certain manufactured chemicals play a vital role in disease prevention and treatment (Quideau et al., 2011). Natural substances are well recognized to possess pharmacological or biotic qualities that might be therapeutically useful for the treatment of cancer, and they also act as a great source of inspiration for the creation of potential novel small molecule medications (Lee et al., 2017; Bernardini et al., 2018). An increasing amount of research shows that natural compounds are essential in the treatment of cancer by inhibiting the proliferation of cancer cells, inducing apoptosis, and halting the spread of tumor cells as well as angiogenesis (Huang et al., 2019). Such substances have an impact on apoptosis, which makes them suitable targets for the Regulated Cell Death (RCD) network (Mittal et al., 2019; Nwonu et al., 2019; Schaaf et al., 2019; Agarwal et al., 2020). Such a substance is geranyl acetate. Geranyl acetate possesses anti-proliferative and anti-cancerous qualities (Mandlik et al., 2022). These monoterpenes provide a fresh therapeutic potential with fewer side effects and occasionally even better efficacy (Ramawat et al., 2009).

MTT assay is versatile which is conducted to evaluate cytotoxicity in which conversion of a substrate to chromogenic product occurs by the live cells only. This assay involves conversion of water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to insoluble purple formazan crystals due to the action of reductase enzymes of mitochondria (Kalsoom et al., 2022). The activity of geranyl acetate was measured by the MTT assay via application of different doses where geranyl acetate showed decreased cell proliferation compared to that in the untreated cells (Figure 2).

Furthermore, trypan blue assay was used to determine number of dead cells after treatment. Live cells do not allow their cell membranes to enter trypan blue dye as compared to the dead cells, hence, dead cells can be easily recognized when their membranes absorb this dye. In the current study, a large number of cells were found dead after treatment compared to those in the untreated group. This shows that this compound may be helpful in killing liver cancer cells. Furthermore, crystal violet staining is also versatile and a quick assay to screen viability of cells after administration of a treatment (Abid et al., 2022). Live cells allow this dye to enter via their membranes, whereas dead cells do not. In this study, the evaluation of viability of cells via the crystal violet assay displayed reduced number of living cells in the treated group as compared to that in the untreated group (Figures 3 and 4).

Induction of apoptosis in cells is a very beneficial strategy to develop anti-cancer agent. Most of compounds derived from plants induce apoptosis via p53 pathway. In the current findings, it was observed that geranyl acetate induced apoptosis in the HepG2 cells via p53 dependent pathway (Figure 5).

Conclusively, geranyl acetate showed a promising anticancer activity in p53 dependent manner and due to its anticancer property, geranyl acetate was able to dysregulate cancer development. This study elaborated anti-cancer capability of geranyl acetate that can be further evaluated at the gene expression level.

Author(s), Editor(s) and Publisher's declarations

Acknowledgement
None declared

Supplementary material
No supplementary material is included with this manuscript.

Conflict of interest
The authors declare no conflict of interest.

Source of funding
None declared.

Contribution of authors

Conceptualization and designing the study: TM. Conduction of experiment: IA. Instrumentation and analysis: I. Preparation of initial draft: FH. Proof reading and approval of the final version: MA.

Ethical approval

For studies involving Human/Animal subjects, a formal ethical approval has been sought by authors from ethical committee/review board of their parent institutions according to their internal guidelines.

Handling of bio-hazardous materials

The author(s) certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discorded to minimize any types of bio-contamination(s).

Availability of primary data and materials

As per editorial policy, experimental materials, primary data or software codes are not submitted to the publisher. These are available with corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Author's consent

All authors contributed in designing and execution of the experiment. All contributors have critically read this manuscript and agree for publishing in IJAEB.

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