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Evaluation of the vanillin treatment on migration and anchorage-independent growth of glioblastoma cell line

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ABSTRACT

Due to the inefficacy of the current treatment regimen, patients with glioblastoma multiforme (GBM), only have a median survival rate of 10–15 months. Temozolomide is the FDA-approved chemotherapeutic agent for the treatment of this lethal disease. A plant-derived compound, such as vanillin (4-hydroxy-3-methoxybenzaldehyde), could be a promising agent for GBM treatment due to its anti-oxidant, neuroprotective, and anti-bacterial activities, and its ability to cross the blood-brain barrier. Vanillin is commonly used as a flavoring agent in beverages and food. The purpose of this study is to explore the effect of vanillin on the human GBM LN229 cell line. The impact of vanillin treatment on cell migration and single-cell colony formation was examined using a wound-healing and soft agar assay, respectively. According to our findings, vanillin inhibited the proliferation of GBM cells. The treatment with vanillin reduced the number of migratory cells and inhibited the capacity of a cell for colony formation in the LN229 cell line. In conclusion, vanillin could be effective in preventing the growth of tumors and might be a potential phytotherapeutic agent for GBM treatment.

1. INTRODUCTION

Glioblastoma multiforme (GBM) is the most lethal type of brain tumor, and patients with GBM currently have a poor median survival rate of approximately 15 months with conventional therapy [1]. The existing standard cure for GBM involves surgical removal of the tumor, after which the patient undergoes radiation therapy and chemotherapy with Temozolomide (TMZ) [2]. Although the current therapy has improved the survival time for patients with GBM, the treatment outcomes are still poor, and almost 90% of glioblastomas will recur within 24 months. [3]. The recurrence of GBM is the major cause of mortality due to the development of resistance to TMZ, which highlights the need to improve the survival of the patient and explore new regimens for GBM treatment [4]. Therefore, it is crucial to develop new therapeutic approaches that can improve the outcomes and protect non-cancerous cells.

Plant-derived substances play a vital role in medical technology. They are an accessible and promising strategy to manage and prevent cancer. This is due to widespread public awareness of the negative side effects of chemically manufactured medications [5]. Vanillin, also known as 4-hydroxy-3-methoxybenzaldehyde, is a phytochemical that possesses various advantageous pharmacological and biochemical properties in this field.

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Vanillin, which is the main ingredient in natural flavor, is used in a variety of foods, beverages, medicines, and cosmetics. It is present in these products at concentrations ranging from 0.3 to 33 mM [6]. Vanillin exhibits chemoprotective effects that include scavenging reactive oxygen species, reducing oxidative damage to lipids and proteins, and lowering the mutagenesis rate [7]. A research study has shown that vanillin decreased the migration and invasion of breast cancer cells [8]. It has also been identified as an anti-proliferative agent in the SH-SY5Y neuroblastoma cell line and hepatocyte HepG2 cancer cell line [9].

In this research, we investigated the impact of vanillin on the cell viability, migration (evaluated by wound healing assay), and single-cell colony formation of LN229 GBM cells.

2. MATERIALS AND METHODS

2.1. Cell Culture

The human GBM LN229 cell line was taken from NCCS, Pune, India and cultured in complete Dulbecco's modified Eagle medium (DMEM) enriched with 10% of fetal bovine serum (FBS) in an incubator set at 37º C with 5% of CO_2.

2.2. 3-(4,5 Dimethythiazol-2yl)-2,5 Diphenyltetrazolium Bromide (MTT) Assay

The effect of the vanillin (purchased from Sigma-Aldrich (V1104)) on the viability of LN229 cells was done using MTT assay as reported by Gautam and Gabrani [10]. The stock concentration (1.6 mM) of

vanillin was prepared in 1X PBS. Further, the working concentrations were prepared by serial dilution in the complete DMEM, ranging from 25 µM to 400 µM. The LN229 cells were treated for 48 h with different concentrations of vanillin. Subsequently, 20 µL MTT solution (5 mg/mL) was added to the cells and incubated for 4 h. DMSO was added to the wells to dissolve the formazan formed in the live cells. The absorbance was obtained at 570 nm using an enzyme-linked immunosorbent assay plate reader. To determine the IC_{50} value, the data from the dose-response curve was plotted on a graph with the concentration of the compound on the X-axis and the percentage of inhibition on the Y-axis. The IC_{50} value was then estimated using the straight-line equation after the X-axis log transformation [11].

2.3. *In Vitro* Wound Area Closure Assay

LN229 cells (1×10^5 cells/well) were seeded and maintained in 6-well plates and kept to grow to a confluent monolayer [12]. Each well was scratched artificially by a sterile pipette tip (200 µL). Subsequently, cells were rinsed with 500 µL of PBS. Following the preparation of the wound area, the cells were treated using vanillin in complete DMEM [13] and kept for 48 h at culture conditions. The wells were rinsed again with 500 µL of PBS, the fresh DMEM was subsequently added, and photographs were captured (at 0 h and 48 h). Evaluation of the wound area closure percentage was performed using Image J software [14]. The effect of the vanillin treatment was assessed by comparing the area closure percentage with untreated (control) cells.

2.4. Soft Agar Assay

The colony-forming study on soft agar was performed to assess a single cell's capacity to form a colony. In a 6-well plate, two agarose layers were prepared, wherein the bottom layer was prepared using 1 mL of 0.6% agarose. The next step was to form the upper layer with 0.3% of agarose, which comprised 1000 GBM cells, untreated or treated with vanillin, for 14 days following the cell culture conditions. The 300 µL of additional media was added to each plate after every three days to prevent dehydration [15]. The colonies were calculated under an inverted microscope to determine the impact of the vanillin on the development of single-cell colonies that are independent of anchoring.

2.5. Statistical Analysis

The analysis of graphed results was conducted using one-way ANOVA with an online tool, the post hoc Tukey HSD test calculator (http://www.astatsa.com). The data for cell viability, wound healing, and soft agar assays have been reported as the mean ± standard error of the mean from three different experiments. Statistical comparisons were made between the cells treated with vanillin and the untreated cells at the $P < 0.05$.

3. RESULTS

3.1. Effect of Vanillin on the GBM Cells Proliferation

Vanillin exhibited concentration-dependent antiproliferative effect in the glioblastoma LN229 cells. The results have shown that 98.67 ± 12.17 µM of vanillin demonstrated 50% reduction in viability percentage (IC_{50}) of LN229 cells [Figure 1] after 48 h of treatment.

3.2. Anti-migratory Effect of Vanillin

We investigated the response of vanillin treatment on migration using area closure or wound healing assay. Figure 2 shows the images of

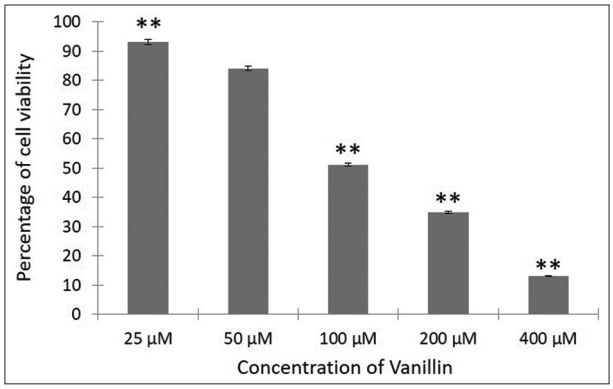

Figure 1: Inhibitory effect of vanillin at different concentrations on LN229 cells. The bar graph represents the viability percentage of LN229 cells after 48 h of treatment. The symbol ** indicates a statistically significant difference ($P < 0.05$).

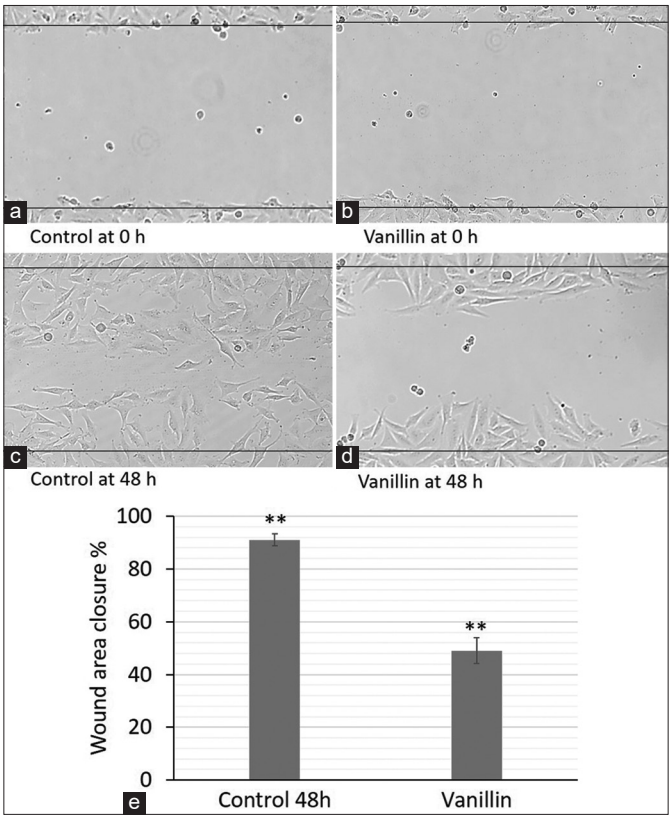

Figure 2: The demonstrative images of wound area closure after the treatment with vanillin on GBM cells in LN229 as examined under an inverted microscope. The panels represent (a) control (0 h); (b) control (48 h); (c) and (d) cells treated with vanillin at 0 h and 48 h, respectively. (e) The wound area closure percentage in cells of control and vanillin-treated cells as evaluated by ImageJ software. The symbol ** indicates a statistically significant difference ($P < 0.05$).

area closure of control at 0 h, 48 h, and treated cells at 0 h and 48 h. The representative control images demonstrated that the wound area closure was almost entirely closed after 48 h [Figure 2b] of incubation as to 0 h [Figure 2a]. To examine the effects of vanillin, the percentage of the wound area closure after 48 h was measured [Figure 2e]. The LN229 cells treated with IC_{50} of vanillin showed ~49% of wound

area closure percentage. This study shows that the vanillin treatment significantly reduced the percentage of migratory cells.

3.3. Effect of Vanillin on Single-Cell Colony Formation

The analysis was carried out to estimate the effect of vanillin treatment on the single-cell colony-forming capability of LN229 GBM cells [Figure 3 and Table 1]. The LN229 cells treated with respective IC_{50} value vanillin exhibited a significant repression of colony formation [Table 1]. The colony percentage was ~58% after vanillin treatment, compared to untreated cells [Figure 3c]. This study demonstrates a significant reduction in the colony-forming capability of the LN229 cells treated with the plant-derived compound vanillin.

4. DISCUSSION

The high frequency of patient mortality amongst those having GBM, complications in the treatment, and failure of current therapy have resulted in the exploration of more novel effective compounds, such as naturally derived substrates, as probable anticancer therapeutics.

Vanillin, a phyto-metabolite originates from essential plant oils, such as *Vanilla pompon* and *Vanilla planifolia* [8]. Across the globe, it is widely used as an aromatic and flavor substitute in foods. The effect of vanillin on growth, wound area closure and anchorage-independent colony formation of GBM cells was examined in this study.

The treatment with vanillin resulted in the restriction of cell proliferation with an IC_{50} value of ~98 µM in the LN229 cells. Although, it has been reported that vanillin reduced cell growth with the 2.6 mM of IC_{50} value in breast cancer cells [16]. In the colorectal cell line (SW480/NNMT), vanillin has been reported to inhibit the cells with 3.15 mM of IC_{50} value [17]. It has been reported that TMZ inhibited the growth of the cells with an IC_{50} value of 452.45 µM in the LN229 cell line [10].

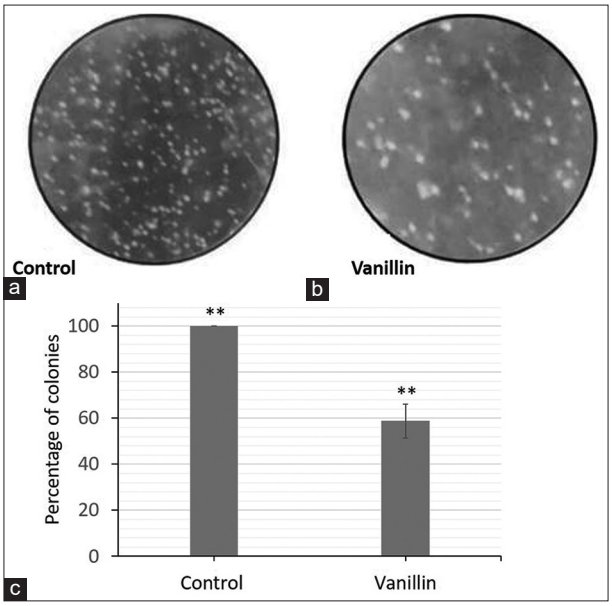

Figure 3: The demonstrative images of soft agar assay after the treatment with vanillin on GBM cells in LN229 as examined under an inverted microscope. The panels represent colony formation by (a) control; (b) cells treated with vanillin. (c) The bar graph represents the percentage of colonies formed by untreated and treated cells. The symbol ** indicates a statistically significant difference ($P < 0.05$).

In this study, the impact of vanillin on migration and colony forming ability of a single cell was also examined. In cancer cells, the addition of FBS can help to create a more *in vivo*-like environment and promote tumor cell migration, invasion, and metastasis. During the wound healing process, FBS can help to induce epithelial-mesenchymal transition (EMT) in cancer cells and promote their migration and invasion [18]. The addition of 10% FBS to the culture media can be important for wound healing assays using cancer cells, as it can help to create a more physiologically relevant environment and promote tumor cell migration and invasion [19].

The wound healing assay is a valid method for assessing cancer cell migration *in vitro*. It is based on the finding that cells proliferating in a monolayer migrate to restore cell connections when an artificial wound is formed [20]. Wang *et al.* described their wound healing assay as examining cell migration in response to a mechanical scratch wound, both in the absence and presence of putative inhibitors [21].

The findings of this study showed that vanillin-treated LN229 cells resulted in the reduction of wound area closure percentage. The vanillin resulted in wound area closure by approx. 49% in the LN229 cells, while treatment with TMZ elicited area closure by 70% [10]. Oliva *et al.* performed the wound healing assay using 10% FBS and showed that isoginkgetin (a flavonoid)- treated cells resulted in the closure of wound area to approx. 25% [13]. In a research study, the human glioblastoma U87MG cells were treated with the IC_{50} value of the respective compound to evaluate the anti-migratory effect by wound healing assay. It was observed that migration was reduced to 60% after the treatment with thymoquinone [22]. Nowicki *et al.* have reported that the downregulation of vimentin, a marker of EMT, significantly decreased the cell migration ability of U87, U251, and U373 GBM cells [23].

The anchorage-independent or detached growth of cells, as an effect of anoikis, induces apoptosis, as a mechanism of action for self-defense used to destroy the detached or misplaced cells [24]. Anoikis, a form of apoptosis that is tempting for a detached cell that is essential for the migration of GBM cells [25]. The soft agar assays are extensively used to estimate anchor-independent cell growth [26]. In the present research study, the vanillin treatment of GBM cells at its IC_{50} value reduced ~42% of the colony formation compared to control Cells. It has been reported that TMZ reduced colony formation to ~20% [10], thus vanillin was effective in limiting the anchorage-independent growth of the LN229 cells.

The treatment with the IC_{50} values of *Thevetia peruviana* fruit methanolic extract is reported to suppress the colony formation and wound healing ability of human colorectal adenocarcinoma (HTB-38), lung carcinoma (HTB-177), prostate adenocarcinoma (HTB-81), and breast adenocarcinoma (HTB-22), cancer cells [27]. It has been reported that vanillin reduced the anchorage-independent growth and colony formation in NCI-H460 lung cancer cells [28].

According to research the treatment with vanillin prompted death for anchorage-independent cells and reduced the formation of colonies in colorectal cells [17].

Table 1: The colony count obtained from the soft agar assay after a treatment period of 14 days in LN229 cells, wherein the symbol±denotes the standard error of the mean.

Treatment	No. of colonies
Control (untreated)	293±19.1
Vanillin	175±32.8

5. CONCLUSION

The finding of the current research demonstrated the antiproliferative and anti-migratory impact of vanillin in the LN229 cells. Additionally, vanillin treatment reduced anchorage-independent cell growth and eliminates the capability of the single cell to grow into a colony. Furthermore, the treatment also improved the wound-healing ability of cells. To conclude, this study suggests that vanillin could be a possible therapeutic agent for GBM.

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7. AUTHORS' CONTRIBUTIONS

R.G. Conceptualization, Review, and Editing of the draft, Resources, and Project Administration. M.G. Execution of experiments and writing of the original draft.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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