Original Article

Anticancer and antiproliferative efficacy of a standardized extract of *Vaccinium macrocarpon* on the highly differentiating oral cancer KB cell line athwart the cytotoxicity evaluation of the same on the normal fibroblast L929 cell line

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Abstract

Background and Objectives: The perpetual search is on to find botanical complementary adjuncts to the conventional therapies used that is not only cost-effective but also reduces side effects associated with conventional synthetic drugs that are available in the market. The aim of this study was to assess the *in vitro* anticancer efficacy of hydroalcoholic fruit extract of cranberry against oral cancer KB cell line by Di-Methyl Thiazoldiphenyl Tetrazolium bromide assay (MTT) assay and its cytotoxicity on normal fibroblast cells.

Materials and Methods: *Vaccinium macrocarpon* extract was prepared using a hydroethanolic solvent (water – 30%:ethanol – 70%) using the standardized maceration protocol. Standard KB and normal fibroblast (L929) cell lines were used. The minimum lethal effect of the extract was calculated using the MTT cytotoxicity assay.

Results: The extract shows a satisfactory antiproliferative effect on the KB cell line and a higher cell viability percentage of the normal fibroblast cell line.

Conclusion: *V. macrocarpon* can prove to be an adjunct to the existing anticancer drug therapy against oral cancer KB cell line.

Keywords: Anticancer, cell viability percentage, cranberry, spectrophotometric determination, *Vaccinium macrocarpon*

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INTRODUCTION

In today's modern world, medicine and science has helped us fight an array of diseases, making the average human life span increase by leaps and bounds. However , this forward path has its cons, targeted through an unhealthy lifestyle, leading to the commencement of a vicious disease non-communicable diseases, i.e., cancer. According to the WHO, "Cancer is the second leading cause of death

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globally, responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 in 6 deaths occurs due to cancer."[1]

The prevalence of cancer in India is estimated to be around 2.5 million, with about 800,000 new cases and 5, 50,000 deaths per annum. In 2003, the Indian Council of Medical Research reported that oral cancer is increasingly common in India. There has been a marked increase in the number of oral submucous fibrosis, especially among youngsters, which further increased the incidence of oral cancer. At present, oral cancer is the fourth most common type of malignancy after lung, stomach and liver in males and the fifth most common cancer after cervix, breast, stomach and lung cancer in females.[2-6]

Although medicine is making strides in the creation of wonder drugs to cure oral cancer, the side effects of these synthetic drugs along with their skyrocketing prices mask their benefits making them unfit for continuous, prolonged. The need for discovering a botanical adjunct to these synthetic drugs to reduce the side effects is the need of the hour. A growing interest has developed to identify dietary components and botanical supplements owing to their therapeutic potential.

Cranberry (*Vaccinium macrocarpon*), the "wonder fruit," has recently come into limelight owing to its significant therapeutic potential. It is a unique and rich source of several classes of bioactive flavonoids, including flavanols, anthocyanins and proanthocyanidins (PACs), which confer it the significant therapeutic potential.[7]

Partly number of studies have been performed on the use of cranberry extract on these strains with a number of pitfalls. The main limitation of the commercially available cranberry extract is the high dextrose and fructose content, which makes it unsuitable for oral use. Further, the presence of methanol in the production of commercially available alcohol, owing to no evidence-based gas chromatography analysis done to discern 100% evaporation of methanol, makes it not favorable as a therapeutic drug.[7] Thus, there is an impending need of assessing the antiproliferative properties of a standard cranberry extract which is versatile and has a wide range of therapeutic uses.

Hence, the current research paper delved into assessing the anticancer potential of *V. macrocarpon* extract on oral cancer KB cell line by MTT assay and the cytotoxicity of *V. macrocarpon* extract on normal fibroblast cells.

MATERIALS AND METHODS

This research was carried out as part and in corroboration with a research done by Kumar *et al*.[8]

Plant materials

Fresh, frozen cranberry fruits (*V. macrocarpon*) (FSSAI FSSL 11214331000541) were accessed from Very Berry Fruits Pvt. Ltd., Bengaluru, Karnataka, India. The fruits were tested and approved by a botanist and pharmacist. They were weighed, rinsed under running tap water and transversely sectioned into two halves. The water/fluid content from the specimens was removed tissue paper blotting. The fresh fruits were dried under the shed (25°C) for 7 days till constant weight was achieved. This technique was done following the method used by Kumar *et al*.[8]

Preparation of crude extract

The dried samples of *V. macrocarpon* were subjected to the maceration method to extract the alkaloids from it. Twenty grams of the dried fruit was macerated with 200 ml of hydroalcoholic solvent, with the ratio of ethanol (70%):water (30%) in a conical flask, plugged with cotton and kept occasionally on a rotary shaker at 190–220 rpm for 48 h at room temperature. The macerated liquid was filtered through Whatman Filter Paper No. 1 (Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India). The extraction processes were carried out for 3 days to obtain crude extracts which were evaporated and concentrated under reduced pressure using a vacuum rotary evaporator (Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India). The residue left was semi-solid, reddish-pink in color, which was soluble in aqueous solution. It was stored at 4°C in an air-tight sterile container till further use.[9,10]

Conditioning of the cell lines

The standard oral cancer KB cell line and the normal fibroblast cell line (L929) were obtained from the National Centre for Cell Science, Pune. They were cultured in a T-flask that was supplemented with Dulbecco's Modified Eagle Media (DMEM), as shown in Figure 1. An estimation of the cell count was done, and it was placed in a carbon dioxide chamber, as shown in Figure 2. The cell counting was done by looking for a change in the suspension color (red to pale), inspection of the surface of the T-flask or using trypan blue exclusion technique using an hemocytometer, as shown in Figures 3 and 4.

Biochemical test

The experiment was carried out using the MTT assay protocol (Di-Methyl Thiazoldiphenyl Tetrazolium bromide assay) [Figure 5].

Cytotoxicity assay

In vitro growth inhibition effect of the test compound was discerned by the characteristic colorimetric/ spectrophotometric determination of conversion of

yellow MTT solution into purple formazan crystals, in the presence of living/viable cells [Figure 6].

Fifty microliters of 1,00,000 cells/ml cell suspension was seeded into each well and DMEM was added, as shown in Figure 7. Following this, dilutions of the *V. macrocarpon* extract were prepared in the DMEM media. One hundred microliters of the extract of different concentrations was added to the wells and incubated for 24 h in the presence of 5% carbon

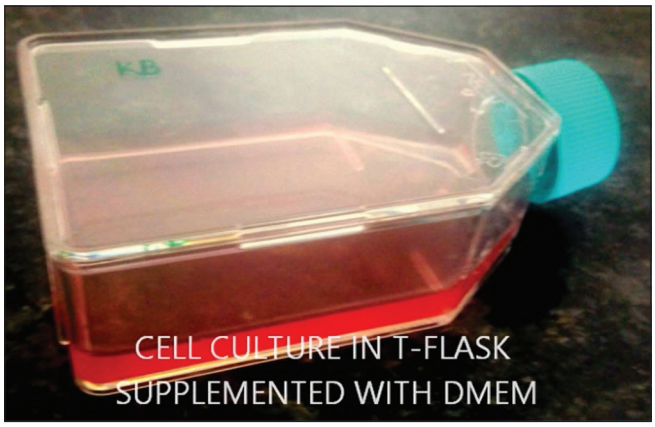


Figure 1: Cell culture in T-flask supplemented with Dulbecco's Modified Eagle Media

Figure 2: Cells incubated in CO_2 chamber

Figure 3: Ten-microliter TB dye + cell suspension

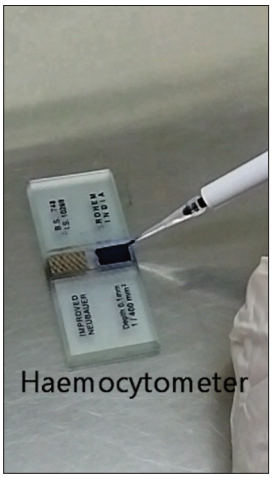


Figure 4: Hemocytometer

Figure 5: Living cells – transparent killed cells – blue

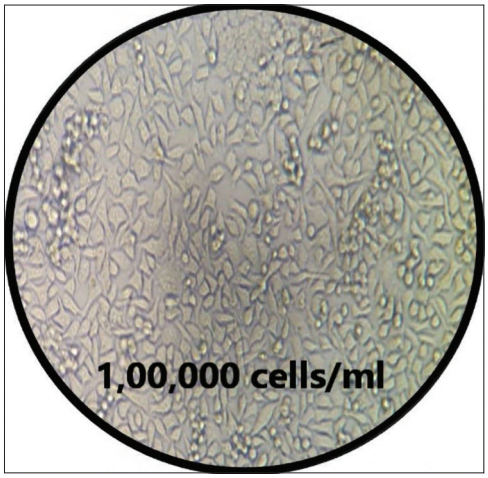


Figure 6: Cell count

dioxide and 37°C in a CO_2 incubator, as shown in Figures 8 and 9. After 24 h, 20 µl of 5 mg/ml MTT reagent was added to the wells [Figure 10]. The plate was covered with aluminum foil since MTT reagent is highly photosensitive [Figure 11]. The plate was reincubated for 4 h [Figure 12]. The supernatant was carefully removed without disturbing the precipitated formazan crystals [Figure 13]. Two hundred microliters of dimethyl sulfoxide was added to dissolve the crystals formed [Figure 14]. Then, the optical density was measured at a wavelength of 492 nm [Figure 15]. The study was performed in triplicates, wherein the result represented the mean value for three readings.

$$\text{Formula}: \text{Surviving cells}\,(\%) = \frac{\text{Mean OD of test compound} \times 100}{\text{Mean OD at control}}$$

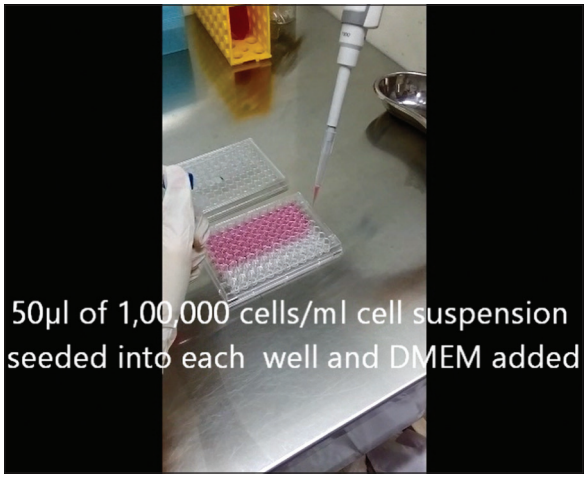


Figure 7: Fifty microliters of 100,000 cells/ml cell suspension seeded into each well and Dulbecco's Modified Eagle Media added

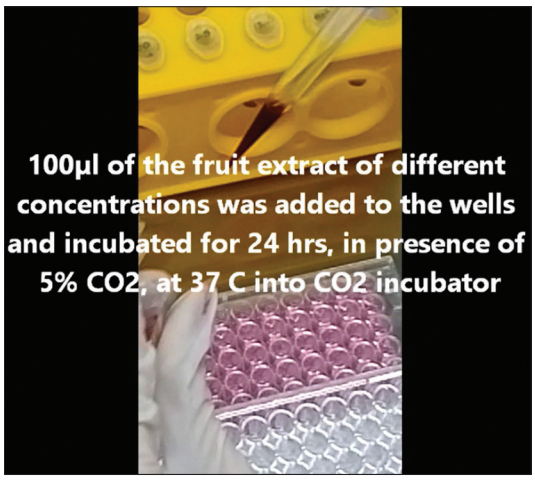


Figure 9: One hundred microliters of the fruit extract of different concentrations was added to the wells and incubated for 24 h, in the presence of 5% CO_2, at 37C into CO_2 incubator

RESULTS

The antiproliferative efficacy of the different dilutions and concentrations of *V. macrocarpon* against the KB cell line and normal fibroblasts is shown in Table 1. As seen in the findings, there is an inverse relation seen with the effect of the extract against the KB cell line with paclitaxel drug as a control and a direct relation with the cell viability percentage of the normal fibroblast cells. Graph 1 shows the activity of the cranberry extract on the two cell strains, giving the percentage of the surviving cells. The result that was obtained after doing an IC50 (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA) was that the dose/concentration of cranberry extract that kills 50%of the oral cancer KB cell line is 3.564 (µg/ml).

DISCUSSION

Scientific evidence strongly suggests that a healthy diet

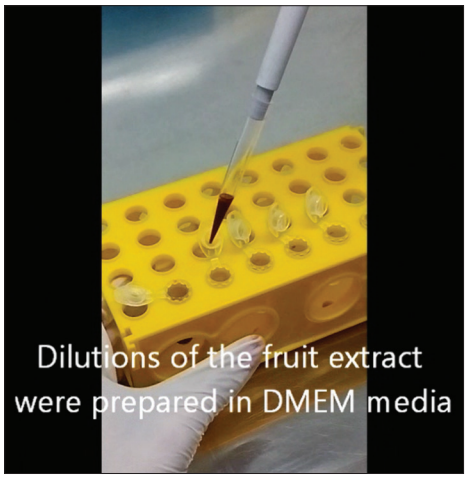


Figure 8: Dilutions of the fruit extract were prepared in Dulbecco's Modified Eagle Media

Figure 10: After 24 h, 20 µL of 5 mg/ml MTT reagent was added to the wells

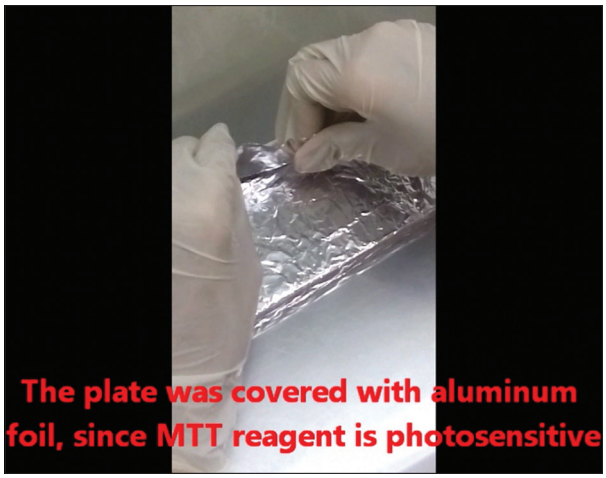


Figure 11: The plate was covered with aluminum foil since MTT reagent is photosensitive

Figure 12: The plate was kept for 4-h incubation

Figure 13: The supernatant was carefully removed without disturbing the precipitated Formazan crystals

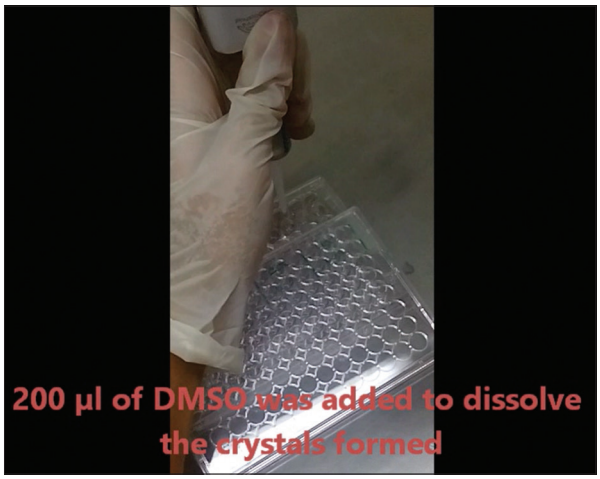


Figure 14: Two hundred microliters of dimethyl sulfoxide was added to dissolve the crystals formed

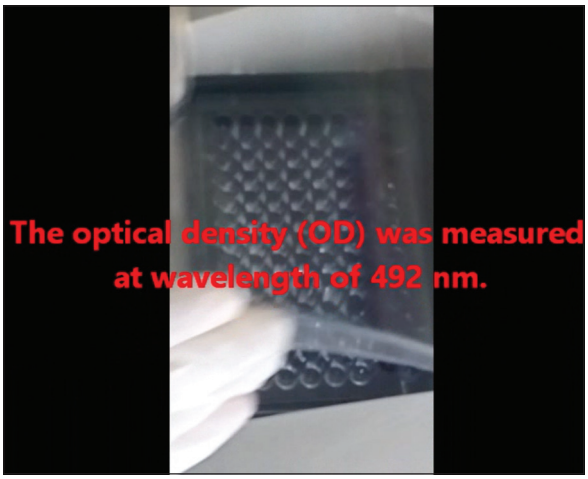
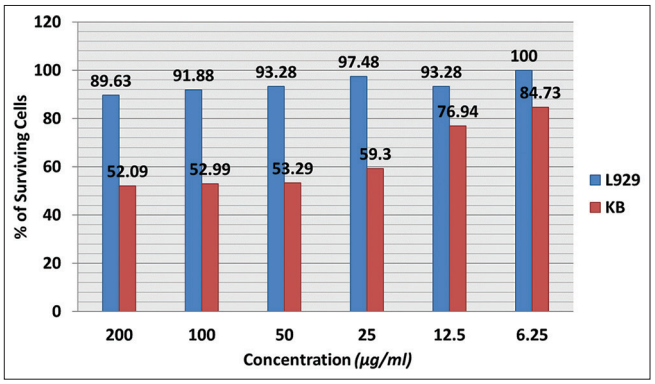


Figure 15: The optical density was measured at wavelength of 492 nmμL



Graph 1: Percentage of surviving normal fibroblast cells and KB cells with different concentrations of the extract

and nutrition plays a vital role in protection against oral cancers.[11] Out of the fruits and vegetables consumed, berries and citrus fruits are highly recommended for oral cancer prevention.[12] For this study, a novel extract preparation of the cranberry fruit was used. Previous studies have used a pure alcoholic extract which was one of their major pitfalls. However, in this study, the

Table 1: Effect of the different concentrations of the hydroalcoholic extract on normal fibroblast cell line and oral cancer KB cell line using paclitaxel as the positive control

Concentration (µg/ml)	Normal fibroblast cell line (L929)		Oral cancer cell line (KB)	
	Mean optical density	Cell viability (%)	Meanoptical density	Cell viability (%)
200	0.319	89.36	0.174	52.09
100	0.328	91.88	0.177	52.99
50	0.333	93.28	0.178	53.29
25	0.348	97.48	0.198	59.3
12.5	0.344	96.34	0.257	76.94
6.25	0.36	100	0.238	84.73
Positive control (paclitaxel)	0.182	51.16	0.144	34.14
Negative control (untreated cells)	0.357	100	0.334	100

incorporation of water in the extract preparation could contribute to the dissolution of the water-soluble active components in the fruit, rendering the wide therapeutic range to the test compound. Studies done by Chatelain *et al.* indicated an affirmative anti-proliferative effect of the cranberry extract on CAL27 and SCC25 cell lines, demonstrating a 34% growth inhibition in CAL27 human oral squamous cell carcinoma (OSCC, GImax = 40 µg/ml) and 36.3% growth inhibition in SCC25 human OSCC (GImax = 70 µg/ml) using the cranberry extract.

Seeram *et al.* further reported the antiproliferative effect of the extract on KB and CAL27 cell line with a similar positive result.[13] The same study also showed that the total polyphenol fraction that was separated from cranberry extract showed 96.1% and 95% of growth inhibition of KB and CAL27 oral cancer cells, which was better than the total cranberry extract.[13] All these studies proved that cranberry extract has some chemoprevention and chemotherapeutic potential against oral cancers and that this could be attributed to the presence of PACs and flavonoids in it.[14] PACs are reported to inhibit the induction of ornithine decarboxylase (ODC), which is an enzyme involved in tumor cell proliferation of epithelial cells.[15]

Another *in vitro* study done by Seeram *et al.* stated that "when cranberries were tested on KB, CAL27, they show no significant antiproliferative action on the cell lines. This could be attributed to the fact that cranberries are known to predominantly contain condensed proanthocyanidins (PACs). These PACs do not present as sharp peaks, rather appear as broad peaks at common HPLC-UV monitoring wavelengths for flavonoids. Hence they are inconspicuous are therefore not visible in the chromatograms (280 nm). Furthermore, the solvent that is used to make the extract is an acidic methanol-based solvent which is more suitable for the extraction of anthocyanins than PACs; therefore, it is unlikely that PACs were present in the extract used for the study."[16]

A study done by the University of Illinois showed that the PACs and other flavonoids contained in cranberry inhibit the ODC activity in mouse epithelial cells (ME-308). ODC inhibition in epithelial cells by cranberry was also linked to its high PAC content.[15]

As stated by a study done, "cranberry extract that is in the form of a water-soluble phenolic extract, prepared from commercially available cranberry powder, can effectively inhibit the proliferation of several human tumor cell lines."[13] A total polyphenol extract containing a variety of flavonoids inhibits the proliferation of two oral cancer cell lines (CAL27 and KB).

The inhibition of tumor cells by cranberry is likely to involve a synergistic activity of the cranberry Phytochemicals, which include the flavonols (quercetin being the major flavonol), PACs and ursolic acid. A few mechanisms of action include induction of apoptosis in cancer cells, decreased invasion and metastasis as a result of inhibition of Matrix Metalloproteinases, inhibition of ODC expression and activity and inhibition of inflammatory processes including cyclooxygenase (COX) activity.[17]

The overexpression of COX-2 plays an important role in promoting certain cancers; therefore, inhibition of its activity or expression is another form of chemoprevention.[17] Inhibition of COX activity by cranberry extracts was noted in a study by Seeram *et al.* in which anthocyanin fractions that were isolated were evaluated for COX-1 and COX-2 inhibitory activity using an assay measuring oxygen uptake on the conversion of arachidonic acid in microsomal preparations by either isoform. Cranberry anthocyanins inhibited COX-1 and COX-2 activity by approximately the same degree, reducing activity by 10% at 125 mg/L.[18]

The results deduced by this research show a decrease in the cell viability percentage of the KB cell. Although it is not as drastic as a result achieved with paclitaxel which is a known chemotherapy drug, the result is still significant enough to attract deeper research in this field. The effect of the cranberry extract on the normal fibroblast cell shows a

high cell viability percentage which discerns lower chances of causing side effects and harm.

On literature search and delving into the methodologies, majority of the experiments documented, though having displayed the anticancer potential of the cranberry extract, have used it on the HeLa cell line of oral cancer make. It is noteworthy that the results yielded from theses studied are guarded and should be interpreted with caution. However, it has been proven that the HeLa cell line is poorly to moderately differentiated cell line for oral cancer.[19] The need of the current study was to target and focus a highly differentiating and proliferating cell line such as the KB cell line.[20]

For use in the oral cavity, we further investigated the effect of the same concentrations of the same standardized extract on the L929 normal fibroblast cells. L929 cell line is widely used for toxicity testing.[21] According to the result of this study, the hydroalcoholic extract of cranberry causes very limited cytotoxicity to this cell line rendering it highly effective in its use against the proliferation of the cancer KB cell line.

Cranberry extract, owing to its botanical origin, can help as an adjunct to the ongoing allopathic drug regimen for patients suffering from oral cancer rather than other synthetic drugs that not only burden the patient with a number of side effects but also have very large monetary implications. The vehicle of the extract was modified to ethanol so as to further reduce any harm that can be caused by the extract.

With further research, *Vaccinium macrocarpon* can be used in various public health and clinical endeavors. This novel extract preparation could be self-administered by incorporating in the formulation of a mouthwash that could be used for independent or supervised rinsing. The cranberry extract has displayed versatility with a broad therapeutic range and low risk-causing profile. Hence it can also be proposed to be administered as systemic metered dose formulation owing to its multiple systemic benefits like its effect against urinary tract infection,[22] cardiac diseases,[23] peptic ulcers,[24] Alzheimer's disease,[25] as an antioxidant[26] and an antiaging agent.[27] They can further be professionally administered as local drug delivery systems such as gels, irrigants, microspheres, nanoparticles, transferosomes or buccal patches directly on oral cancer or precancerous lesions.

We further encourage *in vitro* studies to be performed using this extract to isolate individual components of the extract, time-kill assays and DNA fragmentation procedures and to discern the actual mechanism of the target on cancer molecules through the gene expression or proteomic studies.

CONCLUSION

The study forms a start for extensive and full-fledged research to be carried out with the *Vaccinium macrocarpon* extract. A greater number of clinical trials will improve our knowledge on this wonder fruit. For a dangerous and morbid disease like oral cancer, the authors have strived to present one universal, versatile and standardized solution to act as an adjunct and complementary treatment in the realm of channelized, targeted and palliative care, with predictable patient-centric outcomes.

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Conflicts of interest

There are no conflicts of interest.

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