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# Evaluation of the Anticancer Potential of Whey-Based Beverage Powders Using the MTT Assay

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**ABSTRACT:**

**Introduction**: Oral cancer is a significant source of mortality and morbidity across borders. Therefore, there is a dire requirement to conceptualize groundbreaking protocols that have few negative reactions and proficient efficacy.

**Objectives**: The goal of this study was to use the MTT assay to look at how well a new whey-based drink could fight cancer in KB oral cancer cells, along with its free radical scavenging properties.

**Methods**: The preparation of whey-based beverages involved blending and pasteurizing the ingredients on a lab scale, followed by drum drying. The resulting flakes were ground into a fine powder and stored for further use. The DPPH method was utilized to execute the free radical scavenging activity and cell viability was tested by the MTT assay.

**Results**: Among the three treatment formulations, T2 whey-based beverage powder exhibited better antioxidant activity when compared with the control triphala. Also, the whey-based beverage powders were able to decrease cell survival in KB OC cells.

**Conclusions**: Given that the formulation studied in this research exhibited cytotoxic properties, an *in vivo* approach may provide a more comprehensive investigation of their intrinsic toxicity and cell interactions before their advantageous application.

## 1. Introduction

People's health and well-being are always important issues for both national and international policy. Among the seventeen Sustainable Development Goals (SDGs) set forth by the United Nations and due to be accomplished by 2030, these two components constitute a significant component. To enhance prosperity, the third SDG, "ensure healthy lives and promote well-being for all at all ages," addresses many important factors. Among its related goals are the prevention of non-communicable diseases (NCDs) and a one-third reduction in premature mortality. The load on international health systems is greatly increased by NCDs. As a result, over time, tremendous progress has been achieved in this area to stop and regulate the waves.

One of the four main NCDs and the second-greatest cause of death globally is cancer [1]. On a global and national scale, cancer is still a major public health issue. With rising death rates over time, it is a severe issue that affects the entire population, irrespective of age or

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income. Cancer can be caused by three different types of carcinogens: (i) biological carcinogens, which include hormonal and genetic factors, bacterial, viral, or parasitic infections; (ii) chemical carcinogens, which include contaminated food and water, tobacco use, and radiation from the sun and ionizing radiation; and (iii) physical carcinogens. The primary cause of cancer is thought to be tobacco use, with poor diets coming in second [1].

One of the most prevalent cancers among men in the Indian subcontinent is mouth/oral cancer (OC) [2]. Across national boundaries, OC is a major cause of mortality and morbidity. While surgery is still the most common treatment for OC, there are other choices as well, including radiation, chemotherapy, and other treatments. However, each of these approaches being used has its own drawbacks [3]. Therefore, the key to good cancer management is a multifaceted strategy. Thus, selecting the proper nutritional assistance, particularly in the form of enteral tube feeds, becomes crucial for patients with OC [4]. Thus, it is believed that a crucial supportive element in the overall cancer treatment is appropriate dietary intervention.

There is a growing market for functional foodstuffs and therapeutic beverages as a result of the expanding need for natural products in the food and beverage industries. In the functional beverage market, energy drinks and ready-to-serve beverages are catching on more and more since they are portable and convenient. As a byproduct of the dairy industry, whey has come to be valued as an invaluable repository of protein, lactose, vitamins, and minerals. The general public is beginning to appreciate the nutraceutical advantages that come with whey components, which makes cancer investigations into them a lucrative field. Numerous papers also discussed the vast possibilities of whey proteins (WP) in conjunction with conventional therapies to treat various cancer kinds [5].

## 2. Objectives

Consumer features like convenience, practicality, flavour, and nutritional value affect the size of the whey drink market. Whey and products derived from it can be used to create a variety of beverages with a range of uses and functions [6]. Whey-based drinks, like drinkable yoghurt, flavoured milk, fruit and vegetable-added drinks, etc., play a vital role in this market. Conversely, not much additional study has been done on the cancer-fighting abilities of whey-based beverages on cancer cells. Therefore, the goal of the current study was to examine the antioxidant and anticancer potential of the whey-based beverage powders produced on OC cells by the MTT assay.

## 3. Methods

### 3.1 Preparation of whey-based beverages and powders

The preparation process of these whey-based beverages and powders was submitted for a process patent; hence, the detailed procedure and data were not disclosed. The formulations (T2, T3, and T4) were created through multiple rounds of sensory analysis using whey, tomato pulp, nendran banana, sesame seed, and flaxseed oil cakes sourced from various locations in Mysuru, India. T2, T3, and T4 differed in whey and tomato pulp proportions. The common ingredients included banana, sesame seed, and flaxseed oil cakes. The preparation involved blending and pasteurizing the ingredients on a lab scale, followed by drum drying. The resulting flakes were ground into a fine powder using a mixer grinder and stored in airtight bags at -20°C until further use.

### 3.2 Antioxidant assay of whey-based beverage powders

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method was utilized to execute the free radical scavenging activity of developed whey-based powders, as demonstrated by Blois M with some minor modifications [7]. Samples were added in varying quantities (100 to 300 μg mL−1), to 2 mL of DPPH (100 μM) and properly mixed. The reaction mixture was then made up to 3 mL with methanol and left to sit for 45 minutes at room temperature in the dark. Using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at 517 nm, absorbance was measured at the conclusion of the incubation time against the blank (no sample or standard). The extracts' ability to scavenge free radicals was determined and expressed in terms of IC50 values and compared to triphala, a conventional antioxidant [8]. The percent inhibition was calculated availing the Equation (1):

% DPPH Inhibition = (Absorbance of control-Absorbance of sample)/(Absorbance of control)×100 (1)

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### 3.3 Cell culture and treatment

The cell line assays were performed by making use of the KB OC cell line obtained from the National Centre for Cell Science (NCCS), Pune, India, and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated foetal bovine serum (FBS), penicillin (100 IUmL−1), and streptomycin (100µg mL−1) with pH 7.4, in a humidified atmosphere of 5% CO2, and at 37°C temperature until confluent.

### 3.4 MTT assay of whey-based beverage powders

The cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay [9]. Firstly, confluent cells were trypsinised, and then each well was loaded with 5 × 104 cells in 100 µL and incubated at 37°C for 24 h (5% CO2). The media was removed using a pipette and restored with 100 µL of DMEM containing different concentrations of T2, T3, and T4 whey-based beverage powders as well as triphala powder (served as control) with foetal bovine serum (10%) and incubated for 24 h. Following the treatment period, the medium was discarded from the incubated samples, and 100 µL of MTT solution was added to each well and the samples were then incubated at 37°C for a further four hours. The MTT reagent was removed from the wells, and the formazan salts generated by living cells were then dissolved by adding 100 µL of dimethyl sulfoxide (DMSO). The absorbance was measured at 590 nm using a microplate reader and percent cell viability was calculated according to the Equation (2) [10].

Cell Viability (%) = (Absorbance of sample)/(Absorbance of control)×100 (2)

### 3.5 Statistical analysis

All of the tests were conducted thrice, and the IBM SPSS version 26.0 software program for Windows (SPSS Inc., Chicago, IL, USA) was used to execute the statistical analysis. Mean comparisons by an analysis of variance (ANOVA) plus a post hoc Duncan's Multiple Range Test (DMRT) were applied to assess significance at $p \leq 0.05$.

## 4. Results and Discussion

### 4.1 Antioxidant assay

Antioxidants are vital because they counteract free radical damage. Whey-based beverages powered by tomatoes and other phytosignatures are touted to offer antioxidant abilities. The stable, nitrogen-based free radical, DPPH, is violet in colour. But by taking an electron from the antioxidant molecule, it is reduced to a yellow-coloured diphenyl picrylhydrazine radical that can be quantified colorimetrically. Consequently, antioxidants that are capable of causing this reaction are referred to as radical scavengers [11]. The ability to scavenge DPPH radicals by the whey-based beverage mix was assessed in terms of percent DPPH inhibition and $IC_{50}$ values. The extracts exhibited concentration-dependent free radical scavenging activity and a significant difference ($p < 0.05$) in the DPPH values both between samples and when compared with the control triphala. The scavenger activities of each extract were compared to triphala's scavenging activity, which was used as a positive control for each extract. T2 mix showed maximum DPPH free radical scavenging activity with 60.85% and lowest with 19.58%. T3 reflected maximum activity with 47.89% and the lowest with 16.83%. In the case of T4, the maximum DPPH free radical scavenging activity was 33.85 and the lowest was 12.55%. The graph represented a significant amount of free radical scavenging activity when compared to triphala, where its maximum DPPH radical scavenging activity was 63.42% and its lowest was 20.20%. Thus, among the three, T2 represented the highest DPPH free radical scavenging activity, followed by T3, and then the least could be seen in T4, as shown in Fig. 1. With respect to the $IC_{50}$ values, the T2 mix exhibited a low $IC_{50}$ value of 320.45±9.17µg mL-1, followed by T3 (427.20±7.17) and T4 (616.95±5.38), in which T2 was comparable with that of triphala (Table 1). It is possible that the T2 extract's high concentration of polyphenolic constituents such as lycopene, vitamin C, β-carotene, and other phenolic constituents combined with the ingredients of tomato and nendran banana may contribute to its antioxidant activity when compared to its counterparts with a lower concentration of these ingredients. Multiple research data on antioxidants in extracts that assessed different whey-based beverages' capacity to scavenge free radicals ensured the extracts' ability as antioxidants. A recent study on whey-based bael fruit beverage showed 5.32±0.02 µmol TE/g antioxidant activity by DPPH method, whereas guava-flavoured whey beverage treatments had an antioxidant activity in the range of 1.6-4.6% [12,13]. The DPPH results on whey-based amla

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drinks reported high percent inhibition values ranging from 87.23 ± 0.069 to 93.98 ± 0.076 when compared with the current study findings [14].

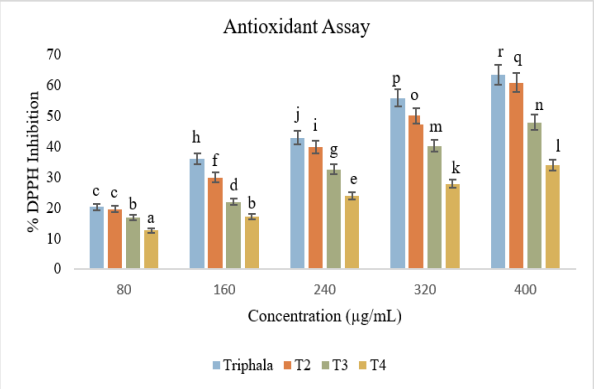



**Fig. 1 DPPH FRSA (%) of whey-based beverage mix**

All values are expressed in Mean±SD. T2- Test formulation 2; T3-Test formulation 3; T4- Test formulation 4. Bars with same letters are not significantly different by DMRT ($p < 0.05$).

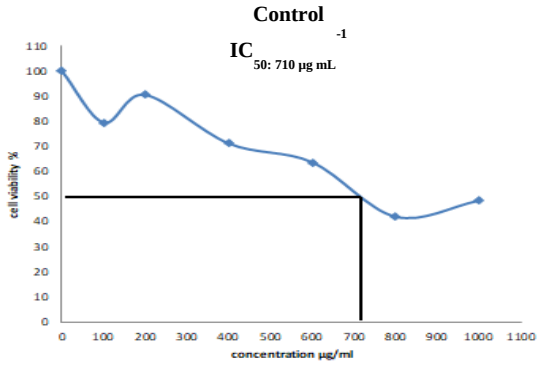
**Table 1. DPPH $IC_{50}$ values of whey-based beverage mix**

| Samples | DPPH activity $IC_{50}$ (µg $mL^{-1}$) |
|---|---|
| Triphala | 279.92± 7.35 |
| T2 | 320.45±9.17 |
| T3 | 427.20±7.17 |
| T4 | 616.95±5.38 |

All values are expressed in Mean±SD. T2- Test formulation 2; T3-Test formulation 3; T4- Test formulation 4.

### 4.2 MTT assay

OC, the sixth most frequent cancer worldwide, is one of the most common types of head and neck malignancies, as the literature has extensively documented [15]. In this specific study, the cytotoxicity of various whey-based beverage powders (T2, T3, and T4) and Triphala was evaluated using the MTT assay, and it was found that the samples showed anticancer activity against KB OC cells. The MTT assay is typically a colorimetric assay employed to determine a cell's metabolic activities, such as cell viability, loss of cell viability (cytotoxicity), etc., wherein living cells transform the MTT yellow salt (water-soluble) into insoluble formazan crystals in the presence of the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxido-reductase enzyme [16]. A solubilization solution like DMSO can be harnessed to dissolve the produced formazan, giving it a purple hue and distinctive absorbance, usually between 500 and 600 nm. The intensity of the purple hue indicates the vitality of the cell and is closely correlated with the quantity of cells. From the findings of the assay, it was observed that cell viability declined with increasing concentrations of the sample. In addition, it was noted that 710 µg $mL^{-1}$ of triphala (control), whereas 490 µg $mL^{-1}$ of T2, 550 µg $mL^{-1}$ of T3, and 800 µg $mL^{-1}$ of T4 were needed to reduce KB OC cells' vitality to 50% ($IC_{50}$) of the starting population at 24 h (Fig. 2). Thus, at 24 h, T2, followed by T3, exhibited lower $IC_{50}$ values against triphala, concluding that formulations T2 and T3 had better cytotoxic activities in the initial 24 h in comparison with the control in the KB cells. These data were comparable to the study results of a recent experiment on probiotic muskmelon health beverage cytotoxicity, reflecting significant decreases in cell viability in the concentrations of 50 µL and 100 µL in the MCF-7 cell line as well as in the concentrations of 25 µL, 50 µL, and 100 µL in the HepG2 cell line [17]. Another study on the antiproliferative and apoptotic effects of probiotic whey dairy beverages on human prostate cancer cell lines (PC-3 and DU-145) also showed cytotoxic activities against both cell lines [18].These data gathered thus point to the deployment of whey-based drinks as a potential source for further cancer research, acting as a means of increasing whey's value too. Further cell line research involving cell cycle and cell death analysis can surely contribute towards affirming the anticancer potential of the whey-based beverage powders under investigation.



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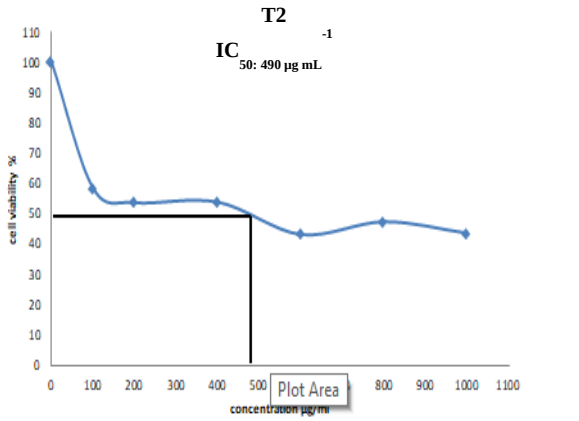


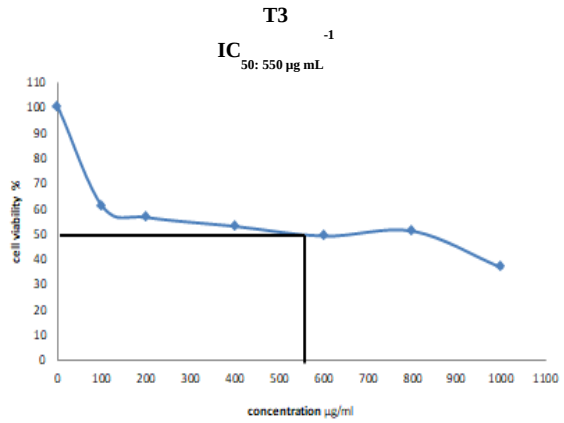


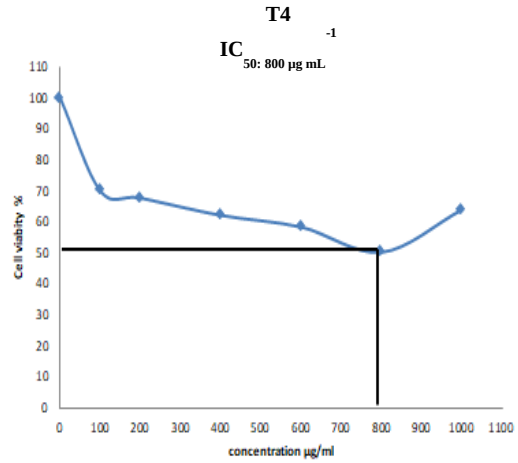


**Fig. 2 Anticancer potential of whey-based beverage mix against KB OC cells using MTT assay**

Control- Triphala; T2- Test formulation 2; T3-Test formulation 3; T4- Test formulation 4.

## Conclusion

The proposed whey-based formulations showed antioxidant and anticancer effects against KB OC cells, according to the investigation's findings when taken as a whole. Additionally, the MTT cell line analysis revealed considerable dose-dependent cytotoxicity towards the KB oral cancer cell line in 24 h. This could have a major impact on the value of whey and further research into the possible anticancer effects of whey tomato-based beverages. To confirm these findings and evaluate the pharmacokinetics and molecular mechanisms behind the observed effects, further cell studies are necessary.

## Declaration of Interest

Authors have no conflict of interest to declare.

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## Author contributions

A.T, M.R and T.V: Conceptualization and design of the product development analysis; T.V: Methodology of the product development analysis; A.T and M.K.P.K: Preparation of product development; A.T, M.R and S.A: Conceptualization and design of the cell line analysis; A.T and A.K: Collected and contributed data, methodology and analysis of cell line data; A.T: Procurement of ingredients for product development, writing and reviewing manuscript; M.R, S.A and M.S.P: Supervision and reviewing the final manuscript.

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