Formulation and evaluation of anticancer potential of whey-based multi-ingredient beverage in oral cancer cell line

A Thesis submitted for the award of

DOCTOR OF PHILOSOPHY

IN

CLINICAL NUTRITION AND DIETETICS

degree based on the research carried out in the Department of

Clinical Nutrition and Dietetics

under the Faculty of Allied Health and Basic Sciences

by

MRS. ANJANA THAMPY., B.Sc., M.Sc.

(Register No: 20PY4001)

Under the supervision of

DR. MADHAVI REDDY., Ph.D.

Professor

Sri Devaraj Urs Academy of Higher Education and Research

(A Deemed to be University, declared under section 3 of UGC Act, 1956)

Tamaka, Kolar, Karnataka - 563 103, INDIA

September 2024

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I, **Mrs. Anjana Thampy** bearing the **Register number: 20PY4001** hereby declare that the work titled **"Formulation and evaluation of anticancer potential of whey-based multi-ingredient beverage in oral cancer cell line"** presented in this thesis is original and does not contain any fabrication. This research work was carried out by me under the supervision of **Dr. Madhavi Reddy** in the Department of **Clinical Nutrition and Dietetics** under the Faculty of Allied Health and Basic Sciences in Sri Devaraj Urs Academy of Higher Education and Research. Furthermore, I have not plagiarized any part of the thesis. Where necessary, I have borrowed material from other authors/researchers and given appropriate credit to them through reference citation or acknowledgement. I also declare that the thesis has not been submitted for the award of any other degree/diploma to any other Higher Educational Institution.

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Date: 06.09.2024

Place: Kolar.

Signature of the Supervisor

06.09.24

Professor
Dept. of Clinical Nutrition & Dietetics
SDUAHER, Tamaka
Kolar-563103

Dr. Madhavi Reddy
Professor
Department of Clinical Nutrition and Dietetics
Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar Karnataka-563103

INSTITUTIONAL ENDORSEMENT

This is to certify that the thesis entitled **"Formulation and evaluation of anticancer potential of whey-based multi-ingredient beverage in oral cancer cell line"** is a bonafide research work carried out by **Mrs. Anjana Thampy**, in the Department of Clinical Nutrition and Dietetics, under the guidance and supervision of **Dr. Madhavi Reddy**, Professor, Department of Clinical Nutrition and Dietetics, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka - 563 103.

Head of the Department

Department of Clinical Nutrition and Dietetics
Sri Devaraj Urs Academy of Higher Education and Research

Clinical Nutrition and Dietetics

Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka-563103

Date: 11/9/24

Place: Kolar

Dean

Dean
Faculty of Allied Health Sciences
Sri Devaraj Urs Academy of Higher Education & Research
Tamaka, Kolar-563 103

Faculty of Allied Health & Basic Sciences

Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka-563103

Date: 12/09/2024

Place: Kolar

Dedicated to my Loving Parents, Husband, Daughter & Brother……

Mr. Thampy Mathew & Mrs. Lisy Thampy

Mr. Arun Lawrance

Ms. Aethel Elizabeth Joseph

Mr. Christy Thampy

For their constant support and care throughout my life……

-ANJANA THAMPY

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Anjana Thampy

Date:06.09.2024 Ph.D. Scholar

LIST OF ABBREVIATIONS

S.NO	Abbreviations	Full forms
01	ALA	Alpha-linolenic acid
02	AOAC	Association of official analytical chemists
03	ANOVA	Analysis of variance
04	βC	Beta carotene
05	BOD	Biochemical oxygen demand
06	BV	Biological value
07	CFTRI	Central food technological research institute
08	CMC	Carboxy methyl cellulose
09	DMRT	Duncan's multiple range test
10	DNA	Deoxyribonucleic acid
11	EAAs	Essential amino acids
12	ESPEN	European society for parenteral and enteral nutrition
13	FOC	Flaxseed oil cake
14	GKVK	Gandhi Krishi Vigyana Kendra
15	GSH	Glutathione
16	HCl	Hydrochloric acid
17	HNC	Head and neck cancer/carcinoma
18	HPV	Human Papillomavirus
19	IIHR	Indian Institute of Horticultural Research
20	IIOR	Indian Institute of Oilseeds Research
21	KOMUL	Kolar-Chikkaballapura District Co-operative Milk Producer Societies Union Ltd.
22	LOH	Loss of heterozygosity

23	LDPE	Low-density polyethylene
24	LDL	Low-density lipoprotein
25	MaPsy	Musa accuminata phytoene synthase
26	NCDs	Non-communicable diseases
27	NPU	Net protein utilisation
28	OC	Oral cancer
29	OSCC	Oral squamous cell carcinoma
30	PER	Protein efficiency ratio
31	PCR	Polymerase chain reaction
32	PUFA	Polyunsaturated fatty acids
33	RNA	Ribonucleic acid
34	ROS	Reactive oxygen species
35	RPM	Revolutions per minute
36	RTD	Ready-to-drink
37	RTS	Ready-to-serve
38	SDGs	Sustainable development goals
39	SDUAHER	Sri Devaraj Urs Academy of Higher Education and Research
40	SDG	Secoisolariciresinol diglucoside
41	SOC	Sesame seed oil cake
42	TPC	Total phenolic content
43	UHPLC	Ultra high-performance liquid chromatography

44	Vit C	Vitamin C
45	WAI	Water absorption index
46	WB	Whey beverage or whey-based beverage
47	WHO	World health organisation
48	WMB	Whey-based multi-ingredient beverage
49	WPs	Whey proteins
50	WSI	Water solubility index

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PLAGIARSIM DIGITAL CERTIFICATE

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH
Tamaka, Kolar 563103

Certificate of Plagiarism Check

Title of the Thesis/Dissertation	FORMULATION AND EVALUATION OF ANTICANCER POTENTIAL OF WHEY-BASED MULTI-INGREDIENT BEVERAGE IN ORAL CANCER CELL LINE
Name of the Student	MRS. ANJANA THAMPY
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Name of the Supervisor / Guide	DR. MADHAVI REDDY
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Signature of Student

Signature of Guide/Supervisor

Professor
Dept. of Clinical Nutrition & Dietetics
SDUAHER, Tamaka
Kolar-563103

University Librarian

Head of the Department

Prof. & HOD
Department of
Clinical Nutrition and Dietetics
Sri Devaraj Urs Academy of
Higher Education and Research
Tamaka, Kolar 563103

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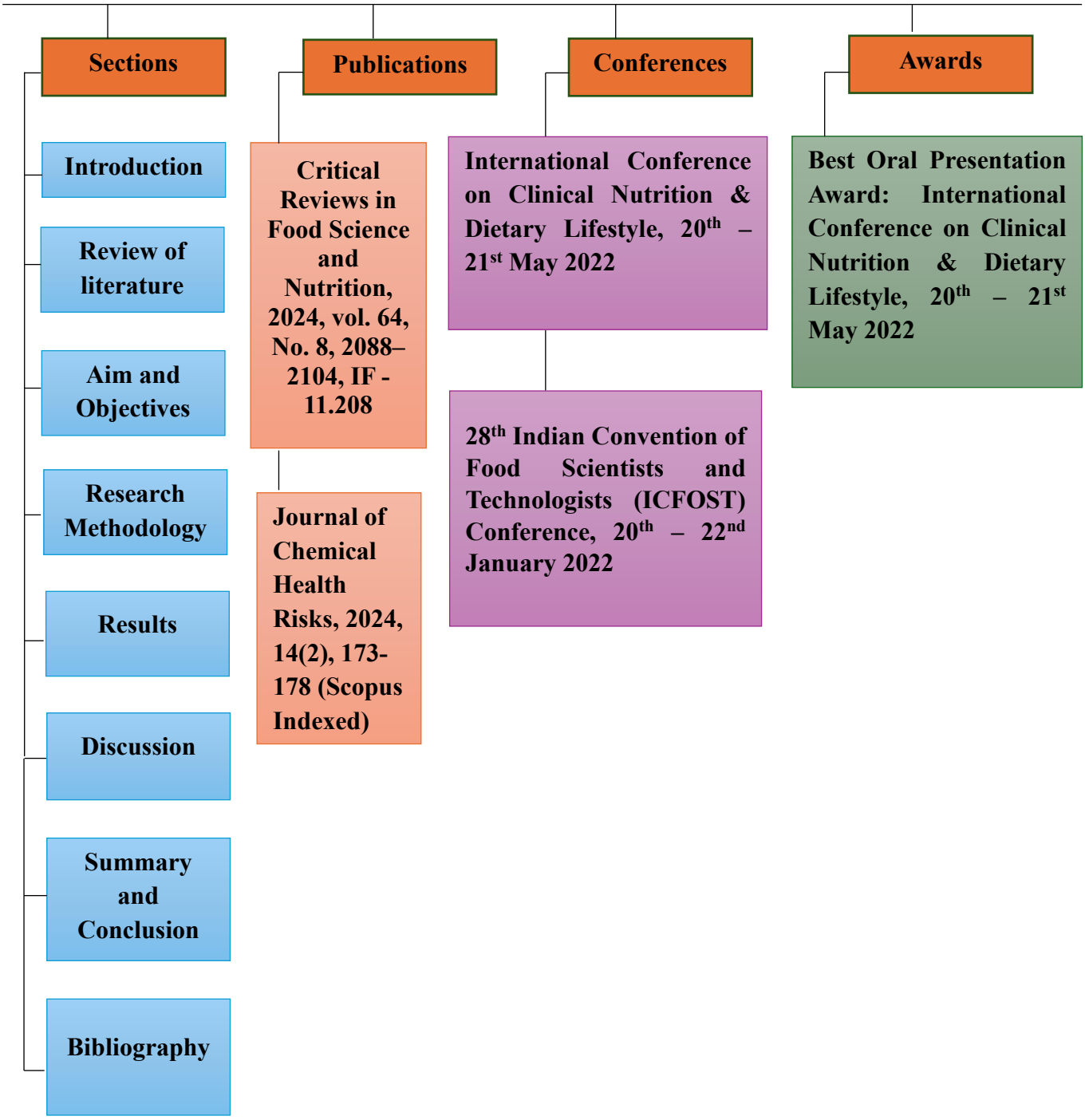
ABSTRACT

Oral cancer is a significant source of mortality and morbidity across borders. The development of novel regimens with high efficacy and minimal adverse effects is therefore desperately needed. The intended purpose of the current study was to formulate a WMB mix by blending whey, tomato pulp, banana, flaxseed, and sesame oil cakes, as well as examine nutritional, physico-chemical, rheological, and sensory aspects along with antioxidant and anticancer potential on KB oral cancer cells by examining its involvement in how it plays a part in cell proliferation, activation of apoptosis, cell cycle arrest, and related morphological changes. Different test formulations were made by varying the amount of whey and tomato pulp in the mixture, then drum drying it. Five variants of drinks were made by blending whey and tomato pulp and labelled as C (0:88), T1 (45:43), T2 (65:23) and T3 (75:13), and T4 (85:3), and each blends have 10% banana pulp, 1% each of flaxseed and sesame seed oil cake. The blend of 45% whey and 43% tomato pulp (T1) presented the maximum overall acceptability scores from sensorial analysis and highest profile in terms of nutritional and bioactive components. It also demonstrated preferable physicochemical, rheological traits and antioxidant profile that could help to valorize whey. The MTT assay revealed that a dosage of 200 μg mL^{-1} was effective for 50% cytotoxicity against KB oral cancer cells but had relatively low cytotoxicity on normal murine fibroblast cells (3T3-L1). Further, T1 at this concentration also induced early apoptosis in the same cell line, whereas standard doxorubicin exhibited late apoptosis. In addition, T1 displayed G2 phase arrest of 25.93% and S phase arrest of 13.07% in KB OC cells that are nearly identical to G2 and S phase arrest caused by the standard doxorubicin. These observations raise the prospects of using whey-based beverages for possible cancer prevention thereby contributing to whey, flaxseed and sesame oil cakes' revalorization.

Key words: Carcinoma, oral cancer, whey proteins, nutraceuticals, nutrients, whey beverages

OVERVIEW OF THE THESIS

FORMULATION AND EVALUATION OF ANTICANCER POTENTIAL OF WHEY-BASED MULTI-INGREDIENT BEVERAGE IN ORAL CANCER CELL LINE



CHAPTER 1
INTRODUCTION

1.0 INTRODUCTION

On a national and global scale, the health and well-being of the populace are perpetually critical concerns. A substantial portion of the seventeen Sustainable Development Goals, or SDGs for short, that were set forth by the United Nations and projected for achievement by 2030 comprises these two elements. The third SDG, which is geared towards "ensuring healthy lives and promoting well-being for all at all ages" to bolster a prosperous future, emphasizes an array of essential facets to augment a better tomorrow. Averting the spread of non-communicable diseases (NCDs) is one of its associated objectives. NCDs drastically hamstring both national and global health systems. Massive strides have been made in this field to control and halt the surges as a consequence.

Based on the latest statistics available for 2022, in the twenty-first century, cancer still stays at the top of NCDs and marks an alarming cause of fatalities, with the number of newly diagnosed cancer cases nearing 20 million, while the mortality toll from cancer drove by 10 million (1). This statistic lends a vivid illustration of the enduring effect that the disease has on communities, producing heightened psychosocial hardship and a drop in overall well-being. It is a perilous malady that exerts substantial societal influence on a large scale. The escalating mortality rates are a critical concern that impacts the entire population, regardless of socioeconomic status or age. All types of carcinogens, a wide range of known and undiscovered determinants, tobacco use, which is the primary root cause of cancer, and faulty dietary practices are just a few of the potential causes of cancer (2).

Oral cancer (OC), a type of head and neck carcinoma (HNC), significantly contributes to morbidity and mortality worldwide, including in India (3). OC is listed as the sixth most prevalent form of cancer on a global scale. India, in particular, exhibits the second-greatest number of OC cases, accounting for nearly one-third of the overall burden (4). India reports approximately fifty-two thousand fatalities and seventy-seven thousand instances that are new

annually, accounting for a quarter of global incidents (4). The Indian workforce is currently decimated by OC. A premature mortality rate of 75.6% was cited in a recent, first-ever study that quantified the decline in productivity in India attributable to premature death caused by OC (5). This rate resulted in a cumulative revenue loss of $5.6 billion, or 0.18% of India's GDP in 2022, as calculated using population-level rates. In a nation where the disparity in affordability is substantial, these findings on the economic ramifications are critical for developing an objective, disease-focused approach to OC treatment. Notably, the study area of Tamaka (Kolar), Karnataka, has a high prevalence of HNC. OC predominates in both genders at 29.6%, which surpasses the national incidence rate of 10.4% and the prevalence rate of 19.59% as reported by Globocan data (6).

OC manifests in the tissues of the oral cavity (commencing from the lips and stretching rearwards to the front section of the tonsils) or oropharynx (the part of the throat behind the mouth) as minor, foreign, enigmatic growths or expansions, encompassing the lips, buccal mucosa (lining of cheeks), salivary glands, teeth, gingiva, tongue, roof (hard palate), floor of the mouth, tonsils, and uvula (4,7,8) (Fig.1). The increasing incidence and mortality of OC are attributed primarily to tobacco use, which is identified as the single most preventable cause of death worldwide (7). Among the risk indicators for the onset of OC other than dependence on tobacco products (including smokeless tobacco and betel quid gnawing) are overindulgence in alcohol, neglected dental hygiene, and protracted illnesses caused by viruses (including human papillomavirus (HPV)) (4). Disparities regarding environmental contact, ignorance, and behaviour-related risk indicators all contribute to substantial variations in global incidence and fatalities.

Though surgery remains the primary treatment option for OC, alternative forms of therapy incorporating radiotherapy and chemotherapy, along with new and underdeveloped immunological treatments, are also in use, but these alternatives are not without downsides,

ranging from therapeutic resistance, potential toxicity, and intolerance (8). Although there has been headway in the treatment of OC, further investigations must be undertaken to comprehend the complex heterogeneity of the disease and to identify the molecular mechanisms that underlie the emergence of aversions to pharmaceuticals and how to combat them to increase patient survival and quality of life, as curative treatments are frequently hindered by the poor nutritional status, general frequent deterioration, and additional ailments that these patients frequently suffer from (8,9). In addition, due to late-phase detection, the prospect of achieving a successful recovery is incredibly shattered and near-negative, and the chance of surviving five years stands at roughly twenty percent (9).

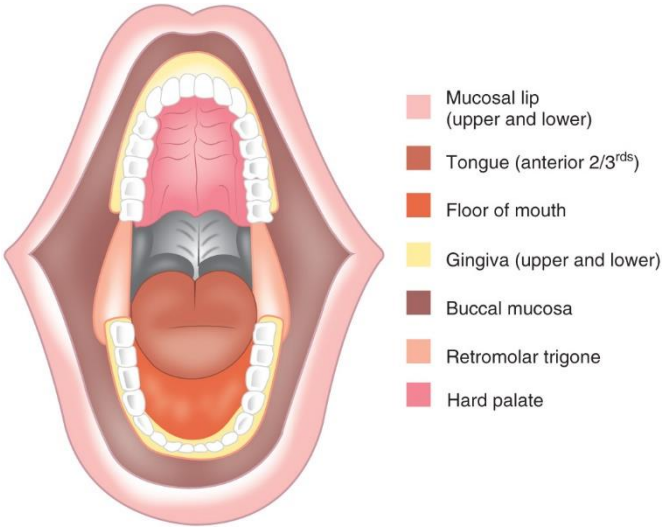


Figure 1. OC spots

Source: Shah JP et al. 2020. Jatin Shah's head and neck surgery and oncology. Chapter 8, 245-297. 5th ed. New York

Natural products are relatively safe for human consumption, which has boosted their popularity across the food industry. While their demand in the food and beverage industries grows by the day, the breadth of functional foods and therapeutic beverages expands as well. Energy drinks and different types of ready-to-serve (RTS) beverages are picking up steam in the functional beverage market, offering tremendous convenience and portability to today's busy customers. As a result, substances that are naturally present have been deemed exciting, key capitals in the

realm of pharmacotherapy that aid in the treatment of numerous non-communicable disorders (10). In addition, a worldwide appeal for foods loaded with protein exists, spanning every age bracket, as healthy eating gains traction (11). In this context comes the significance of whey and its products, which were once thought to be waste products but are now widely employed within the current health drinks and restorative dietary enterprises. It is a by-product of the milk coagulation process that is used to make several dairy products, such as caseinates, cheese, curd, and paneer, among others, with a plethora of industrial functionalities (12). It results from the coagulation of dairy proteins by enzyme action, lactic acid bacterial fermentation, or acidification of milk (13,14).

The nutraceutical benefits of whey components are becoming more extensively recognized by consumers, making cancer research on them an appealing area. Whey contains highly biologically valuable proteins, water-soluble vitamins, minerals, and lactose. It is a powerhouse of nutrients, having good protein quality scores with a biological value (BV) that exceeds that of an egg (15). Whey proteins (WPs) are endorsed as wonder proteins due to their impeccable essential, sulphur-containing, and branched-chain amino acid profiles, which are vital in tissue development and repair as well as glutathione (GSH) formation and have found widescale use in the clinical nutrition and dietetics domains, particularly in supplementary feed formulations (15–17). In addition, they also offer exquisite protein efficiency ratios (PER), net protein utilisation (NPU), quick digestibility, assimilability, and solubility, along with excellent functional properties (15,18). Besides, WP, with its role in muscle building and repair, is perhaps the type of protein that attracts the most attention among sportspeople and has held a prominent position among protein powders in the sports nutrition sector for ages (19). Various articles also addressed the massive potential of WPs when used in tandem with standard treatments to treat different types of cancer (11). However, according to recent research developments, whey's benefits go even beyond muscular anabolism and have shown several

positive health effects involving antioxidants, anticarcinogenic, anti-inflammatory, anti-bacterial, and antiviral properties, immune modulation, gastrointestinal health improvement, etc. (20).

Because of the massive amount of whey generated (~9 L from 1 kg of cheese), the milk products industry occupies one of the most environmentally detrimental sectors (21). Whey disposal is an intense environmental concern owing to its escalated biochemical oxygen demand (BOD) (22). Utilization or valorisation of whey, on the other hand, may be beneficial not just to the environment but also to a long-term economic strategy (23). Apart from being an outcome of cheese production, whey is now utilized in the creation of a broad range of meals and additives, comprising pharmaceutical items, food supplements, fermented foods, beverages, semisolids, and so on. Whey beverages (WBs) employing vegetables and fruits are lately garnering massive emphasis as their economic possibilities expand. The therapeutic effect of whey will be enhanced by the addition and synergistic action of well-established antioxidant natural ingredients from tomato, banana, flaxseed and sesame seed oil cakes for cancer patients. However, evidence on the role of disease-specific dietary interventions with whey and other ingredients is limited. Literature thus demands evidence from basic research to human intervention studies to establish its therapeutic effects in the Indian population. In light of the necessity for robust anticancer agents and the connection of these ingredients' intake with lesser cancer risk, a current study was executed integrating all these components that are very common and easy to get and to assess their anticancer potential in OC.

1.1 NEED FOR THE STUDY

Despite the multifaceted health benefits and the huge potential for whey solids to be used in drinks, whey is still underutilized in the diet of common people. Although a wide range of WBs, including plain, carbonated, alcoholic, and fruit and vegetable juice additions, have been fruitfully produced and distributed worldwide, the majority of these products are primarily intended to quench thirst, and the therapeutic possibilities of enrichment with other phytochemical-rich dietary components have not been fully explored and are poorly documented. The therapeutic effect of whey will be enhanced by the addition and synergistic action of well-established antioxidant natural ingredients from tomato, banana, flaxseed and sesame seed oil cakes for cancer patients. The antioxidant and anticancer roles of bioactive elements from these ingredients have been extensively evaluated and established across the world in managing the severity of cancer.

In addition, Kolar, popularly known as milk city, is famous for its milk production unit, “Kolar-Chikkaballapura District Co-operative Milk Producer Societies Union Ltd. (KOMUL)”, which is “Karnataka’s second highest milk producing district organization”. It provides a wide range of quality milk and milk products to the public. However, due to the lack of a processing unit, large quantities of whey are disposed of as waste during cheese manufacturing, which in turn poses a serious environmental threat due to its high BOD. Further, according to the Indian Horticultural Database (2011), Karnataka is the nation's top producer of tomatoes, with Kolar being one of the leading suppliers to surrounding areas and neighbouring states (24). Unfortunately, 20–40% of tomatoes are not used in India due to inadequate transportation and storage facilities (25). Of all the carotenoids, lycopene, the primary pigment found in tomatoes, has the highest level of antioxidant activity and it also exert significant anticancer properties (26). Bananas are packed with functional, health-promoting nutrients such as vitamin C (Vit C), provitamin A carotenoids, potassium, magnesium, calcium and fibre along with antioxidant

enzymes such as ascorbate peroxidase, catalase, peroxidase and superoxide dismutase all of which have significant implications in the prevention of cancer (27–29). In specific, *Nendran* banana has reported the greatest beta-carotene (βC) content amongst the major Indian banana cultivars (30). Additionally, the nutrient-dense defatted meal made from flaxseeds and sesame seeds is not fully utilized for human dietary consumption; rather, it serves as livestock feed or is disposed of as refuse to a large extent, even today. Flaxseed oil cake (FOC) is abundant in protein, fiber, lignans, total phenolic compounds, and most importantly, alpha-linolenic acid (ALA) content (31). Further, the FOC lignans have been linked with lesser risk of certain cancers due to their antioxidant, anticarcinogenic, antimutagenic, anti-proliferative, antiangiogenic, anti-invasive, antimigratory, and antiestrogenic properties along with induction of cell death (32). Sesame seed oil cake (SOC) is also a protein-rich byproduct with profound sulphur containing amino acids and unsaturated fatty acids profile with wide range of health promoting effects including anticancer properties (31). Thus, it is now imperative to find new applications for whey, tomatoes, banana and agricultural by-products of sesame seeds and flaxseed oil cake.

Above all, and most importantly, there is a high prevalence of OC in Kolar. While comparing with the high national prevalence of 19.59%, it is 29.6% in Kolar, which signifies the immediate need to address the issue (6). Besides, the anticancer potential of whey or whey with other food ingredients on OC cells, however, has not been the subject of any additional research. The need for the study has evolved from this research gap, and hence, this work was undertaken to formulate whey-based multi-ingredient beverages (WMB) adopting whey along with other natural ingredients for the goal of obtaining health benefits at a minimal cost as well as to investigate nutritional, physicochemical, sensory, and anticancer potential behaviours.

CHAPTER 2
REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

Subsequent sections present a literature review concerning OC prevalence, aetiology, clinical presentations, pathophysiology, hallmarks in OC, whey and its significance in cancer, role of tomatoes, bananas, FOC, and SOC in cancer, utilization of whey in beverages, and so on.

2.1 OC

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2.1 OC

2.1.1 OC prevalence and aetiology

OC, a type of HNC, significantly contributes to morbidity and mortality worldwide, including in India (3). OC is listed as the sixth most prevalent form of cancer on a global scale. It is a massive worldwide public health matter, as evidenced by the 1.40-fold spike in the mortality rate associated with malignancies of the oral cavity and lip over the last thirty years (33,34). OC has a 45% rate of mortality in five years of diagnosis (considering all phases of diagnosis combined), according to an estimate by the World Health Organization (WHO) (35). India, in particular, exhibits the second-greatest number of OC cases, accounting for nearly one-third of the overall burden (4). It is one of the most ingrained forms of the disease in India, making it a growing priority for society as a whole, as roughly seventy percent of reported cases are in the end stages. India reports approximately fifty-two thousand fatalities and seventy-seven thousand instances that are new annually, accounting for a quarter of global incidents (4). Indeed, nearly one-third of the worldwide incidence and mortality associated with OC occurs in India, and the treatment is frequently morbid, causing impairments in swallowing, respiration, and communication, with a high probability of premature disease recurrence. A very recent OC study aimed at quantifying the productivity decline in India attributable to early death resulting from OC presented a premature mortality rate of 75.6% that incurred a cumulative revenue loss of $5.6 billion, or 0.18% of India's GDP in 2022, as determined by population-level rates (5). These results are crucial for developing an objective, disease-focused strategy for OC care in a nation where the affordability disparity is substantial.

All types of carcinogens, a wide range of known and undiscovered determinants, tobacco use, which is the primary root cause of cancer, alcohol intake, and faulty dietary practices are just a few of the potential causes of cancer (2). The increasing incidence and mortality of OC are attributed primarily to tobacco use, which is identified as the single most preventable cause of

death worldwide (7). Tobacco and tobacco products are liable for over 27% of all cancer cases in India (5). Despite the fact that smoking-related mortality for cancers of the oral cavity and lip has fallen significantly over time, cigarette smoking still continues to account for as much as one-third (30.5%) of deaths associated with these malignancies worldwide (34). An abundance of study data currently confirms that tobacco use, particularly conventional (i.e., combustible) and e-cigarette use, drives up the likelihood of acquiring various types of carcinomas, with particular emphasis on tumours affecting the lips and oral cavity (34). While certain countries have made progress in reducing the negative health effects of smoking through the implementation of recent smoking dissuasion policies, further healthcare interventions must be planned immediately to alleviate the substantial global burden of cancers affecting the oral cavity and lips caused by cigarette smoking. This is particularly true in regions where the burden is greatest, such as Europe and the Western Pacific, compared to Southeast Asia (34). In India, tobacco use commences at an exceptionally young age, which contributes to the high incidence of oral cancer at an early age. Notably, the study area of Tamaka (Kolar), Karnataka, has a high prevalence of HNC. OC predominates in both genders at 29.6%, which surpasses the national incidence rate of 10.4% and the prevalence rate of 19.59% as reported by Globocan data (6).

Among the risk indicators for the onset of OC other than dependence on tobacco products (including smokeless tobacco and betel quid or areca nut gnawing) are overindulgence in alcohol, neglected dental hygiene, protracted illnesses caused by viruses (including HPV), family and past history of cancer, extreme sun exposure, compromised immune system and nutritional deficiency (4,36). Besides these, subjects' age has a direct correlation with the incidence of OC. Following the age of 40–49, OC rates increase markedly before plateauing between 70–79 years (37). Also, males are more susceptible to OC than females, and in accordance with its anatomical site in the oral cavity, the incidence of the condition in males is

two to six times that of females chiefly due to their greater consumption of tobacco and alcohol (37). Disparities regarding environmental contact, ignorance, and behaviour-related risk indicators all contribute to substantial variations in global incidence and fatalities. The heightened prevalence of OC observed in the study area of Tamaka (Kolar), Karnataka can be attributed specifically to the increased intake of alcoholic beverages and tobacco (6). To summarize, OC's disease process is influenced by multiple factors, including economic, environmental, and systemic influences (Fig. 2) (37). The occurrence of this disease is ultimately determined by the interaction of these variables.

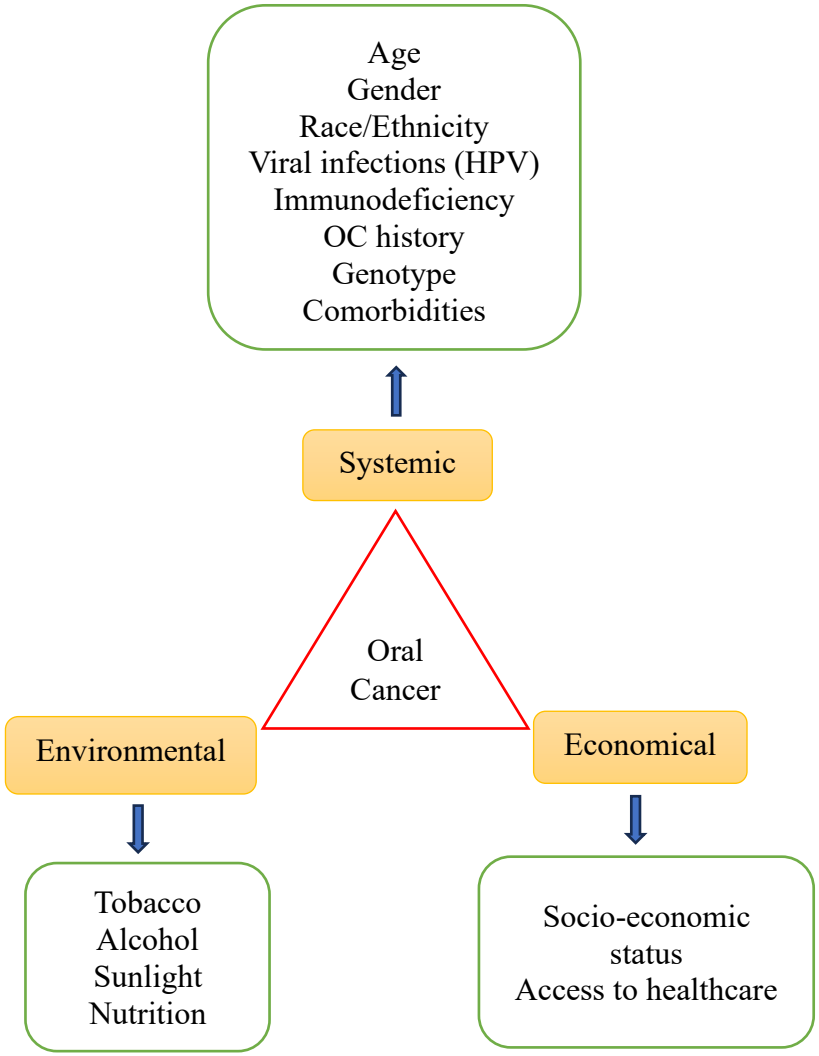


Figure 2. OC risk factors

Source: "Jones DL, Rankin KV: Oral cancer and associated risk factors. In Cappelli D, Mobley C [eds]: Prevention in clinical oral health care, St Louis, 2008, Elsevier, pp. 68-77".

2.1.2 Clinical presentations of OC

Distinctive OC patient concerns may encompass “oral ulcers or masses, swallowing and breathing difficulties, odynophagia, voice hoarseness, globus sensation, otalgia, stuffiness in the ear or sinus, haemoptysis, trismus, neck mass, and head/neck pain,” etc. Among the signs and symptoms that are more general in nature are fatigue, anorexia, mood swings, and disturbed sleep. On a clinical level, oral malignancies may manifest as “erythroplakia (a flat red patch) that may resemble traumatic or inflammatory lesions, leucoplakia (a white patch), raised lesions, ulcerated lesions, and warty lesions or growth” (38). On the one hand, patients often present with discomforts such as pain in the mouth, inadequate movement of the tongue, difficulty opening the mouth, and mastication, leading to dysphagia and decreased appetite. This consequently results in poor compliance with oral feeds and poses a high malnutrition risk, adversely affecting the overall treatment prognosis (39,40). On the other hand, the tumour, the responses of the host to the tumour and the treatment itself (side effects) usually end up in malnutrition (41,42). Several other factors, including psychological stress, also contribute to cancer-related malnutrition (43). As a result, patients’ daily nutritional requirements are not met satisfactorily, leading to depletion of nutritional status, rapid weight loss, and nutritional complications. Sophisticated and challenging to address, malignancy-related malnutrition is rendered more so by the multifaceted cancer cachexia, which is marked by negative energy balance, skeletal muscle loss resulting from decreased consumption of meals, and metabolic disturbances (44). A holistic approach therefore lies at the heart of effective cancer management.

2.1.3 Oral carcinogenesis

OC pathogenesis, or oral carcinogenesis, is a multifaceted and intricate phenomenon that necessitates an in-depth comprehension of the concepts governing the spread and progression of tumour. It requires a deep understanding of its molecular attributes, including genetic and epigenetic modifications, as well as the intricate signalling pathways that are stimulated and often interact (45). This knowledge is crucial for developing new diagnostic molecular markers and personalized therapy for oral premalignancy and cancer. Similar to other forms of cancer, OC also develops gradually, with healthy epithelium undergoing a series of developmental phases that culminate in the development of invasive phenotypes. While all varieties of carcinomas can be observed in the regions of the oral cavity and lip, oral squamous cell carcinoma (OSCC) forms the predominant type of OC. Genetic as well as proteomic approaches are being used to uncover the molecular pathological profile of OC, focusing on oncogenes, genomic instability, and gene expression patterns. However, an exhaustive understanding of OC pathology and its correlation with underlying factors requires further research (46).

2.1.4 Phases of oral carcinogenesis

OC, and OSCC in particular, is a complex, multistage process distinguished by unique epigenetic and genetic modifications. Invasive OC develops as a consequence of a progressive accumulation of molecular aberrations, such as epigenetic modifications, chromosomal defects, and mutations. These defects cumulatively trigger a phenotypic shift from healthy epithelium to dysplastic tissue and ultimately to invasive OC (37,45) (Fig. 3). While there's a general progression from normal tissue to cancer, it's not always a straightforward process; the order and number of mutations can vary, and some stages may even be skipped. While dysplasia (precancer) is generally a precursor to OC, it's not always necessary for severe dysplasia to occur before cancer develops, as cancer cells sometimes appear before severe

dysplastic changes. This complex process by which epithelial cells acquire invasive properties entails their interaction with connective tissue components and the immune system. As cancer cells progress through subsequent genetic modifications, their capacity to invade, metastasize, and elude immune system eradication is enhanced (45).

Monoclonality in carcinogenesis pertains to the process by which "all cancer cells originate from a solitary progenitor that initially experienced genetic aberrations." Tumour evolution (TE) refers to the progression of a single cell alteration in healthy tissue into the formation of a tumour mass. Intratumor heterogeneity (ITH) arises when genetically divergent subclones differentiate within the mass of the tumour. The literature identifies four primary TE theories: linear, branching, neutral, and punctuated (45).

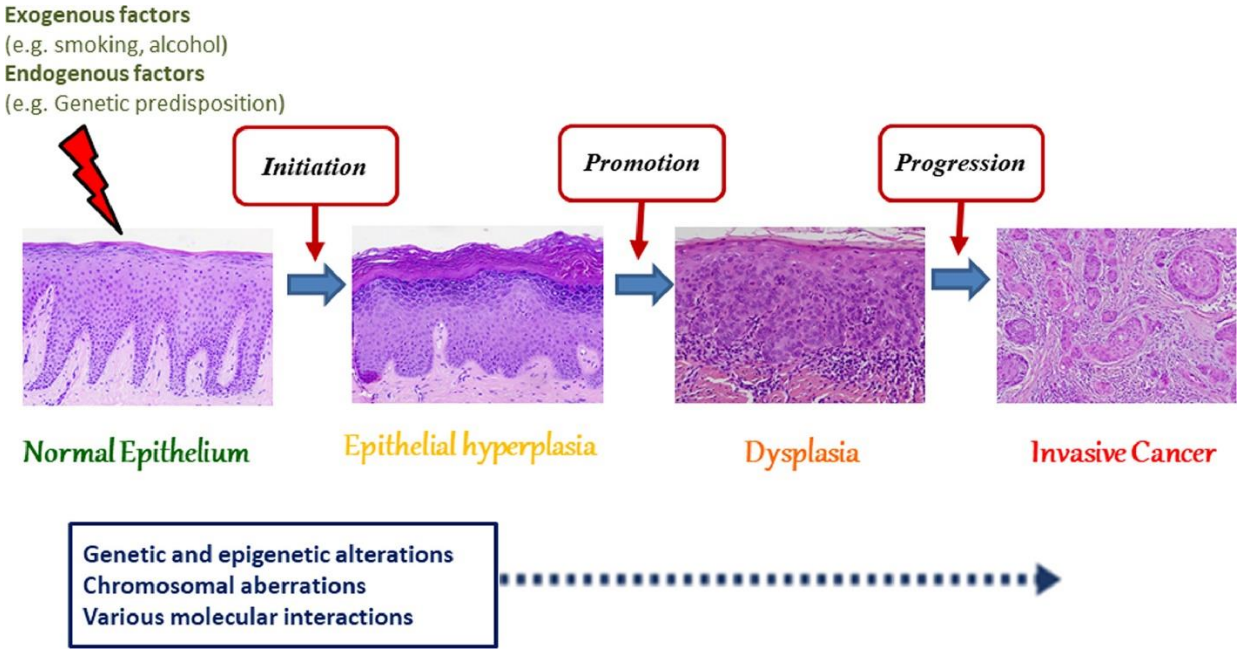


Figure 3. Progression of OC

Source: "Georgaki et al. 2021. Understanding the complex pathogenesis of oral cancer: A comprehensive review, p- 566-579" (45)

Once the initial clone has been established, subsequent mutations facilitate its continued existence and growth as a clonal neoplastic population. The aberrant proliferation of clonal cells can be attributed to their capacity to elude deoxyribonucleic acid (DNA) repair

mechanisms and immune recognition. Field cancerization, which occurs when genotypically clonal cells replace adjacent normal keratinocytes, results in an elongated "field" that may surpass the boundaries of observed lesions at the clinical and microscopic levels (45). This phenomenon complicates the management of potentially deadly and malignant conditions, further escalating the incidence of recurrences or secondary primary malignancies.

Cancer development at a molecular level involves activating tumour-promoting signals and inhibiting tumour-suppressive ones. Genes known as oncogenes and tumour suppressor genes regulate these processes. They also influence how cancer cells interact with their surroundings, promoting blood vessel growth, inflammation, and immune system evasion. The mechanisms behind oncogene activation and tumour suppressor gene inhibition are complex and variable, involving chromosomal aberrations, mutations, and epigenetic modifications (37).

Oral carcinogenesis is particularly affected by loss of heterozygosity (LOH) and aneuploidy. It is monitored for chromosomal abnormalities through the utilization of “molecular techniques such as polymerase chain reaction (PCR), fluorescence in situ hybridization, and karyotype analysis.” High frequencies of “LOH and microsatellite instability” are observed in oral mucosal lesions that have the potential to become malignant. “Defects in chromosomal regions 9p21, 3p14, and 17p13, which contain genes implicated in malignant transformation,” are linked to dysplastic lesions. Aneuploidy, characterized by chromosomal number anomalies resulting from chromosomal instability, exhibits a positive correlation with both the extent of dysplasia and the likelihood of malignant transformation (8,37,45).

Epigenetic mechanisms, such as posttranscriptional silencing, DNA methylation, and histone modifications, exert an influence on the regulation of gene expression. A significant method for modulating the “expression of genes implicated in oral carcinogenesis is the methylation of promoter regions.” Genes that are hypermethylated have been identified in oral dysplastic lesions. The packaging of chromatin may be altered by posttranslational modifications that

affect histones, thereby influencing the expression of genes associated with oral cancer. The prominence of ribonucleic acid (RNA)-mediated epigenetic modifications, including those "mediated by microRNAs (miRNAs or miRs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs)," has increased in recent times. MicroRNAs (miRNAs) are single-stranded RNAs that influence cell differentiation, apoptosis, proliferation, and other processes through interactions with DNA, RNA, or proteins. OC (OSCC) tissues contain an abundance of lncRNAs that display aberrant expression levels, characterized by either upregulated expression (onco-lncRNAs) or downregulated expression (tumour suppressor lncRNAs). Circular RNAs, which are produced through the process of back-splicing a precursor mRNA, have the capability to be translated into proteins and regulate gene expression to different degrees(8,45).

Recent research provides further evidence for the "differential expression of numerous circRNAs, some of which have been identified as being dysregulated" in other forms of cancer. While some circRNAs exhibited upregulated expression in oral cancer samples relative to control tissues, expression was downregulated in OSCC. A potential mechanism by which CircANTRL1 contributes to the increased radiosensitivity of OC is by inhibiting miR-23a-3p, which in turn increases PTEN expression (8,45).

A multitude of signalling pathways undergo dysregulation during oral carcinogenesis, resulting in the excessive activation of oncogenic pathways. "The mammalian target of the rapamycin (mTOR) pathway, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), Ras-Raf-mitogen-activated protein kinase (MAPK), and Janus-kinase/signal transducer and activator of transcription (JAK/STAT)" are all significant pathways (8,45). Oral premalignancy and cancer have been associated with EGFR overexpression, which induces the "recruitment and activation of oncogenic molecules including RAS, PI3K, and Stat3." These pathways demonstrate substantial cross-talk, primarily via positive regulation, which supports the notion

that downstream synergistic cascades produce tumour-promoting functions after the upstream event occurs. Overexpression of the JAK/Stat3 pathway occurs during the earliest phases of oncogenesis, which is a crucial factor in the disease progression of oral cancer. In addition to modulating cellular proliferation and apoptosis through its function as a transcription factor, Stat3 interacts with NF-kB and MAPK via IL-6 (8,45).

2.1.5 Hallmarks implicated in OC

Below is a description of the redefined hallmarks of cancer implicated in oral carcinogenesis, with a depiction of the same in Fig. 4.

2.1.5.1 Deregulations in cell cycle and proliferation control

"Deregulations in cell cycle and proliferation control" are required for carcinogenesis, which calls for the appropriate management and regulation of checkpoints. Cyclins and other positive regulators facilitate the transition between phases of the cell cycle, whereas negative regulators impede its progression. Changes in these regulatory factors have been identified in OC and precancerous lesions, whereby a considerable proportion of lesions exhibit elevated cyclin D1 levels. Cell proliferation biomarkers are disrupted when cell cycle control is lost during carcinogenesis; in healthy mucosa, the number of cells in the proliferation phase ranges from 20% to 45% in dysplastic lesions and up to 60% in OSCC (45).

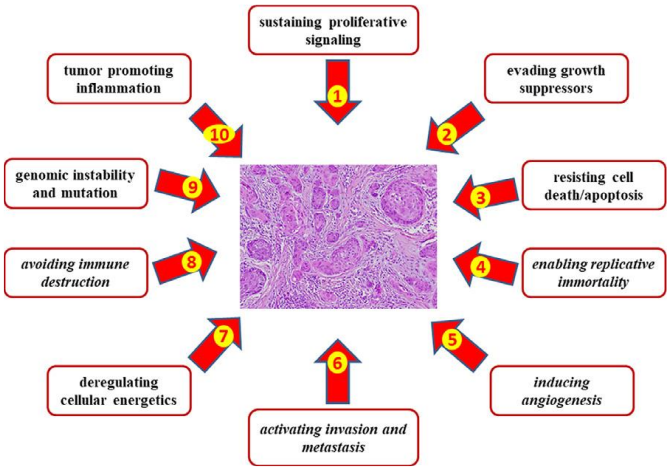


Figure 4. Redefined hallmarks of cancer implicated in oral carcinogenesis

Source: "Georgaki et al. 2021. Understanding the complex pathogenesis of oral cancer: A comprehensive review, p- 566-579" (45)

2.1.5.2 Evasion of apoptosis

Apoptosis is an essential process for the elimination of damaged or aging cells. Disruption of apoptotic pathways alters the balance between cell death and proliferation, leading to an excessive proliferation of cells, which is a major factor in the development of cancer. In OC, alterations in the expression of apoptotic molecules, including survivin and Bcl-2 family proteins, are frequently observed. Dysplastic oral lesions and OC were found to have an increase of Bcl-2 and Bcl-X proteins (antiapoptotic), indicating their potential role in the development of OSCC (47).

2.1.5.3 The regulatory function of p53 and pRb as tumour suppressor molecules in cell proliferation and apoptosis

The transcription factor p53, sometimes referred to as the tumour protein 53 (TP53) gene, functions as a tumour suppressor by regulating apoptosis, cell cycle progression, differentiation, and DNA repair, among other processes. “Dysregulation of the p53 tumour suppressor gene's activity” is a common molecular phenomenon in cancer and malignancies of the mouth. It aids in the removal of cells that are prone to malignant transformation by causing apoptosis or stopping the cell cycle to allow DNA repair. It can intervene in antitumor mechanisms, activate repair of damaged DNA, and initiate apoptosis. Some pathogens, like the HPV, directly affect p53, leading to rapid cell death or block of cell division. It encodes a protein that binds to and deactivates p53. This, in conjunction with the inhibition of p105RB, an additional cell cycle regulator, facilitates the recurrent cell divisions observed in the clinical manifestation of the wart. It has been demonstrated that the introduction of p53 into protein-deficient cells expedites the demise of cancer cells or halts cell division. Occurring in 50% of tumour types, p53 is a well-researched biomarker for the oral cavity that is mutated in 25–69% of cases of OSCC. In 40–67% of HNC patients, high p53 expression is seen. While some scientists emphasize p53's involvement in the cancer process, others have found a direct

correlation between the upregulation of this protein and a bad prognosis for survival. Since inactivated p53 is unable to halt DNA damage-related cell division, OC may begin. Cells are deprived of a crucial regulatory mechanism that prevents carcinogenesis when p53 is rendered inactive by “mutations, loss of heterozygosity, or MDM2-mediated degradation.” p53 immunohistochemical expression is significantly elevated in both oral malignant and precancerous lesions. The Rb pathway is important in controlling the course of the cell cycle. Loss of the Rb pathway is observed in 66% of instances of OSCC and 64% of premalignant lesions. Survivin, an apoptotic process inhibitor, is also a possible marker of OC (47). In addition to tumour suppressive properties, the retinoblastoma protein (pRb) impedes the uncontrolled advancement of the cell cycle. Perhaps pRb or p16 inactivation might foster the development of OC (45).

2.1.5.4 Potential for unlimited proliferation

Cancer cells possess a defining characteristic in their capacity for perpetual multiplication, unaffected by aging mechanisms or programmed cell death. The aforementioned characteristic, known as “immortalization, is intrinsically linked to the aberrant operation of telomerase, an enzyme-containing protein that aids in the maintenance of telomere length”. Due to the fact that “cellular senescence results from the normal reduction in telomere length that occurs with each cell division, telomerase activity that is excessive can result in cell immortalization, thereby contributing to increased invasiveness and oncogenic activity”. In addition to OC, precancerous lesions of the mouth have exhibited telomerase activation, which has been linked to an elevated risk of malignant transformation. The primary etiological factors, tobacco and alcohol use, induce cellular damage leading to compensatory hyperproliferation and an elevated probability of unrepaired DNA damage. Across multiple generations of cells, this injury gives rise to a precancerous lesion or aberrant clone known as leucoplakia. While leucoplakia is reversible, additional harm will inevitably lead to the development of OC (48).

2.1.5.5 Angiogenesis

Angiogenesis, which involves the proliferation and migration of endothelial cells to generate new blood vessels is an essential characteristic of malignant neoplasms. Tumours stimulate angiogenesis in order to supply cancer cells with oxygen and nutrients, thereby enhancing their capacity for metastasis. Angiogenesis favours pro-angiogenic factors over anti-angiogenic ones during carcinogenesis, leading to an increase in micro vessel density. Angiogenesis is significantly influenced by factors such as “vascular endothelial growth factor (VEGF) and nitric oxide synthase 2”, whereas OSCC is characterized by decreased levels of anti-angiogenic factors, including endostatin and angiostatin. Tumour growth requires substantial blood supply for metastasis, requiring proangiogenic and antiangiogenic signals. OSCC has a high invasive capacity and local invasive capacity, with a high predisposition to metastasize in cervical lymph nodes. These processes involve cell adhesion, cytoskeletal rearrangement, migration, basement membrane degradation, passage and survival in the bloodstream, and colonization with new vessels (47).

2.1.5.6 Invasion and metastasis

The oral mucosa's epithelial cells have the ability to invade, rupturing the basement membrane and multiplying inside the connective tissue. This change allows cancer cells to enter the bloodstream and allows the infiltration of the underlying tissues, which distinguishes OSCC from precancerous lesions. Invasion and metastasis of cancer cells are dependent on alterations in cell adhesion and motility molecules, in addition to the epithelial-mesenchymal transition program (45).

2.1.5.7 Inflammation

By facilitating cancer invasion and dissemination via growth signals and microenvironment modification, inflammation functions as an essential factor in carcinogenesis, eradicating cancer cells via immune surveillance. In HNC, neoplastic cells exhibit heightened

concentrations of cytokines and NF-kB, which are molecules associated with inflammation. Over the course of several cancer types, the chemo-preventive efficacy of COX inhibitors and nonsteroidal anti-inflammatory medications has been studied (45).

2.1.5.8 Evasion of immune system

To avoid being recognized and destroyed by the immune system, cancer cells evolve strategies for defence, such as "direct inhibition of antigen recognition and suppression of immune cells, chiefly cytotoxic T lymphocytes". Through the production of soluble chemicals, OSCC cells develop traits that provide them resistance to T lymphocytes and indirectly affect immune system function. Natural killer cells, M1 macrophages, and CD8+ T lymphocytes all function poorly in patients with OSCC, whereas other immune-suppressive cell types show improvement (45). A depiction of disruption of major biologic functions in oral carcinogenesis is given below in Fig. 5.

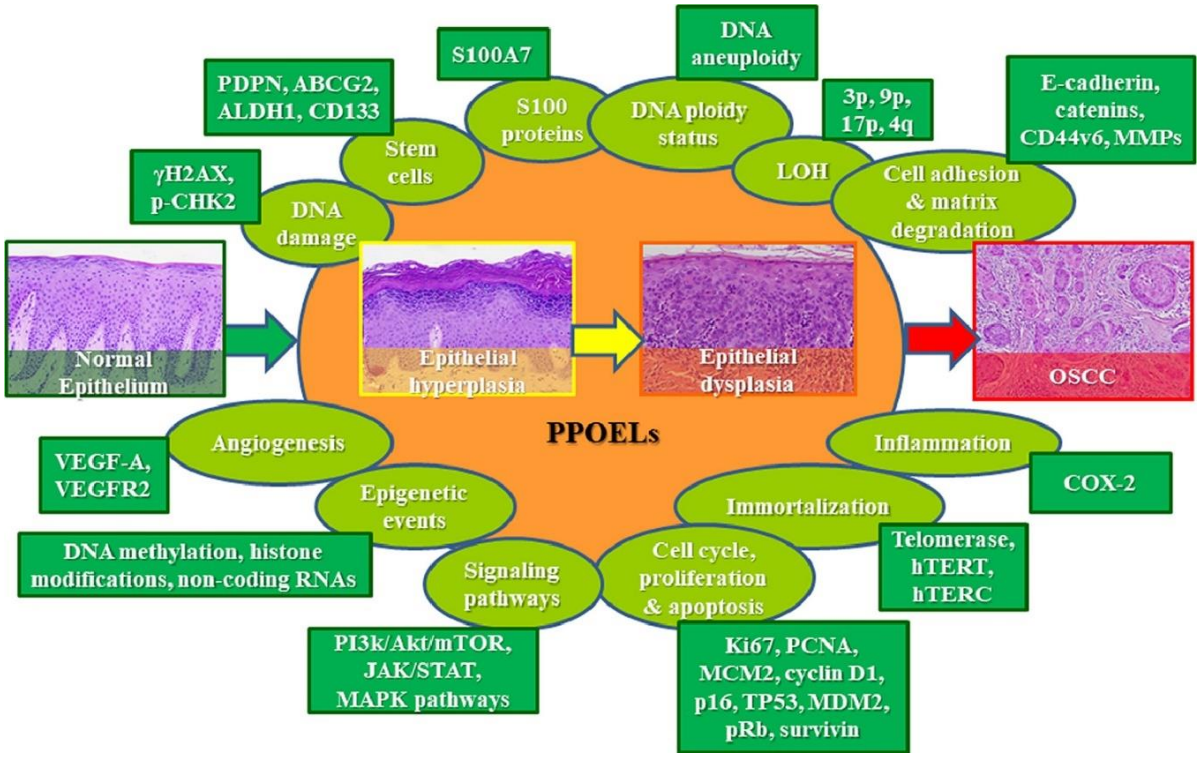


Figure 5. Depiction of disruption of major biologic functions in oral carcinogenesis
Source: "Georgaki et al. 2021. Understanding the complex pathogenesis of oral cancer: A comprehensive review, p- 566-579" (45)

2.2 Whey

2.2.1 Composition of whey

Whey's makeup and features are impacted by the purity and source of the milk used and the processing techniques used to create the final product (17,49). Whey has 20% of the total milk protein content and contains approximately 50% of the nutrients in milk, primarily lactose (14). After water, the chief ingredients of both acid and sweet whey are minerals, WPs, and lactose. It is primarily comprised of approximately 93–94% water, 4.5–5.0% lactose, 0.7–0.9% soluble protein, 0.6–1.0% mineral ions, and substantial amounts of B vitamins (14). WPs serves as the most invaluable element of whey among these and comprises beta-lactoglobulin (61.4%), alpha-lactalbumin (20.5%), lactoferrin, and blood proteins; serum albumin (6.0%); and immunoglobulins (IgG1, IgG2, IgA, and IgM) (12.2%) (50). The bioactive non-protein whey components are lactose and lipids (sphingolipids) (50). Table 1 below represents the composition of Indian cheese whey.

Table 1. Composition of Indian cheese whey

Constituent	**Composition (%)**
Moisture	93.52
Total Solids	6.48%
TSS	11.2° Brix
pH	6.21
Lactose	5.00
Total Protein	0.78
Fat	0.26
Ash	0.59
Calcium	291 mg/L
Sodium	260 mg/L
Potassium	1300 mg/L

Source: Nupur Goyal and D.N. Gandhi 2009. Comparative Analysis of Indian Paneer and Cheese Whey for Electrolyte Whey Drink. World Journal of Dairy & Food Sciences 4 (1): 70-72 (51).

2.2.2 Components of whey

Whey, a by-product of milk protein coagulation, can be sweet or acid whey, subjected to the setting during which it gets treated (52). Sweet whey, which is the most prevalent and greatly utilized in the majority of WP powders, is a “byproduct of rennet-induced coagulation of milk proteins, which results in hard cheeses such as mozzarella, cheddar, and Swiss cheese,” and “acid whey (sour whey), a by-product generated during acid coagulation of milk proteins, which results in products such as cottage and other fresh cheeses or strained yogurt” (52). Scholarly research has reported and established that the whey fractions possess the ability to be applied as a “nutraceutical in the therapeutic sector”. Whey also contains many kinds of non-essential trophic elements that are effective in enhancing health and fighting disease, as indicated by the increasing body of evidence (50). Despite the fact that all of these elements are highly beneficial in terms of their impact on health, WPs, which make up 20% of the total protein content of milk, showcase varied biological action in terms of functionality, with an average of 4 to 7 g protein/L (14,53). They are considered "wonder proteins" as a result of their multifaceted biological effects (16). Among the most vital WPs are “beta-lactoglobulin (β-lactoglobulin–BLG), alpha-lactalbumin (α-lactalbumin–ALA), lactoferrin (LF), glycomacropeptide (GMP), immunoglobulin (Ig), bovine serum albumin (BSA), and lactoperoxidase (LPO) enzymes” (54,55). A few kinds of natural growth factors have also been noticed in mitogenic bovine whey extract, including “transforming growth factor-β (TGF-β), insulin-like growth factor I and II (IGF-I and IGF-II), platelet-derived growth factor (PDGF), and fibroblast growth factor 1 and 2 (FGF-1 and FGF-2)” (56). “Hydrolases, transferases, lyases, proteases, and lipases” are among the varieties of enzymes that make up in whey (55). A detailed description of the various WP components was reviewed recently by Thampy et al., 2024 (11).

2.2.3 Whey's nutritional profile

Because consuming a healthy diet is becoming more and more popular, people of all ages are seeking “high-protein food” items. The Recommended Dietary Allowance (RDA) states that an adult in good health should consume “0.8 g of protein per kilogram of body weight per day (g/kg/day)” (57). The human system demands this amount of protein in order to maintain a “healthy nitrogen balance and to operate at peak metabolic efficiency”. In view of the cancer nutrition perspective, oncology-related malnutrition is far harder, intricate, and tougher to address due to the “multilayered cancer cachexia, which has been marked by negative energy equilibrium, skeletal muscle atrophy brought on by decreased nutritional intake, and metabolic abnormalities” (41). Employing the right kind of dietary guidance remains vital for sufferers of cancer, since optimal dietary management is seen as a key therapeutic dimension in the entire oncology tactics (44,57). Providing “premium protein-rich formulations comprising essential amino acids (EAAs)” is seen as a great option for dietary assistance, especially for “cachexia to stimulate muscle protein synthesis (MPS), in the treatment of cancer” (44,57). According to the standards set forth by the “European Society for Parenteral and Enteral Nutrition (ESPEN),” patients with cancer should aim for a daily protein consumption of “at least 1 g/kg and, if feasible, up to 1.5 g/kg” (44). When compared to other dietary proteins, WP possesses “antioxidant properties, the top protein quality rating qualities, and all of the EAAs,” making it an excellent choice in this scenario (16,50,58). As quoted above, WPs were renowned as “wonder proteins” (16). “EAA profile, protein efficiency ratio (PER), biological value (BV), simple digestion, assimilability, and solubility” are the reasons why WPs are touted as “wonder proteins.” The optimum amino acid pattern exists in WPs, which are exceptionally high in “sulphur-containing amino acids (methionine and cysteine) and branched-chain amino acids (leucine, isoleucine, and valine)” (15). These amino acids are crucial for “glutathione (GSH) biosynthesis as well as tissue growth and repair” (17). Besides, Renner (1992) documented that

“WPs have greater BV (104) compared to casein (77) and whole eggs (100). They also have better PER and net protein utilization (NPU) than casein.” “While the NPU is 92 for WPs, 76 for casein, and 94 for whole eggs, the PER of WPs is 3.6 compared to 2.9 for casein and 3.8 for whole eggs (15)” (Table 2). Besides, since whey contains the “highest concentration of readily absorbed and digestible EAAs,” it can be integrated into body cells more quickly than other dietary protein sources and improves plasma amino acid and protein synthesis (59). An extra advantage of leucine for cancer cachexia therapy is its capacity to induce MPS at lower doses. WP dietary supplementation is also an intriguing feeding approach that has demonstrated effectiveness in cell line, animal, and human interventional investigations (60).

Additionally, they exhibit basic utility features that allow for a variety of purposes in dietetic foods and beverages as well as powders, like isolates, and protein concentrates. In the case of cow's milk protein intolerances, WP hydrolysates as well as whey-predominant formulations have been widely used in newborn nutrition. Recent studies are focused on biologically active peptide sequences from whey, which are essential for immune-boosting effects and enterohormone secretion. All these traits make whey valuable in nutritional applications, particularly for oncology patients' specific feed formulations. Table 2 below compares whey's protein values to those of other foods.

2.2.4 Whey's role in cancer

WPs have been researched extensively in the treatment of cancer due to their bioactive components (61). “GSH stimulation, antioxidant activity, apoptosis induction, iron binding capacity, regulation of cell growth, suppression of cell proliferation, and stimulation of MPS are the key mechanisms reported in several review articles for its antitumor and anti-carcinogenic activity” (11). GSH plays a vital function in protecting cells from UV radiation, poisons, infections, free radical damage, and pollution. Moreover, cancer patients often have

reduced GSH levels and a weakened immune system (17,61). Thus, providing methionine and cysteine-rich WPs can be a good therapeutic option as they are utilised for GSH synthesis. By producing oxidative damage to the nucleic acid structure and consequent oxidative damage to tissues, free iron functions as a mutagenesis promoter. The ability of lactoferrin present in whey to bind iron is thus an extra advantage in cancer nutrition as they induce apoptosis of tumour cells (17,61). Sphingomyelin, which is a whey-derived sphingolipid, is proven to inhibit colon cancer (17). Numerous animal studies have demonstrated the ability of bovine lactoferrin to treat various cancers, including colon cancer (62,63). Similar findings are also reported from research pertaining to various malignancies, including those of the tongue, oesophagus, lung, and bladder (63,64). In addition, some recent clinical studies reported a positive correlation of WP supplementation (WP isolate and WP concentrate form) with the nutritional status of malnourished cancer patients (65–67). Mazzuca et al. 2019 reported the benefits of WP supplementation in improving the nutritional status of colorectal cancer patients and preventing chemotherapy-related toxicity (67). Another study by Bumrungpert et al. 2018 also presented the desirable outcomes of "improved nutritional status, glutathione levels, and immune function in cancer patients undergoing chemotherapy" (65). Similar positive results of "improved body composition, muscle strength, and reduced chemotherapy-induced toxicity" were shown by Cereda et al. 2019 in "malnourished advanced cancer patients with chemotherapy" (66). Taken together, it can be summarized that "when a cancer patient consumes WP, the amino acids stimulate MPS and also result in an increased rate of apoptosis, a decrease in GSH concentration, and cell proliferation in cancer cells, as well as increased expression of IGF-1 by liver cells, which all contribute to the cytotoxicity of cancer cells" (11) (Fig. 6).

Table 2. Comparison of protein values of whey with other foods

Food Protein	BV	PER	NPU
Whole Egg	100	3.8	94
Egg white (Albumin)	88	-	-
Cow's Milk	91	3.1	82
Casein	77	2.9	76
Beef	80	2.9	73
Potato	71	-	-
Soya	74	2.1	61
Rice	59	2.0	57
Wheat	54	1.5	41
Beans	49	1.4	39
Lactalbumin/WP	**104**	**3.6**	**92**

Source: Renner E 1992. Nutritional aspects in Whey and Lactose Processing. Whey and Lactose Processing edited by J.G. Zadow, Elsevier Applied Science London. Elsevier Science Publishers Limited England, Springer Science & Business Media 1992; P. 449-471 (15).

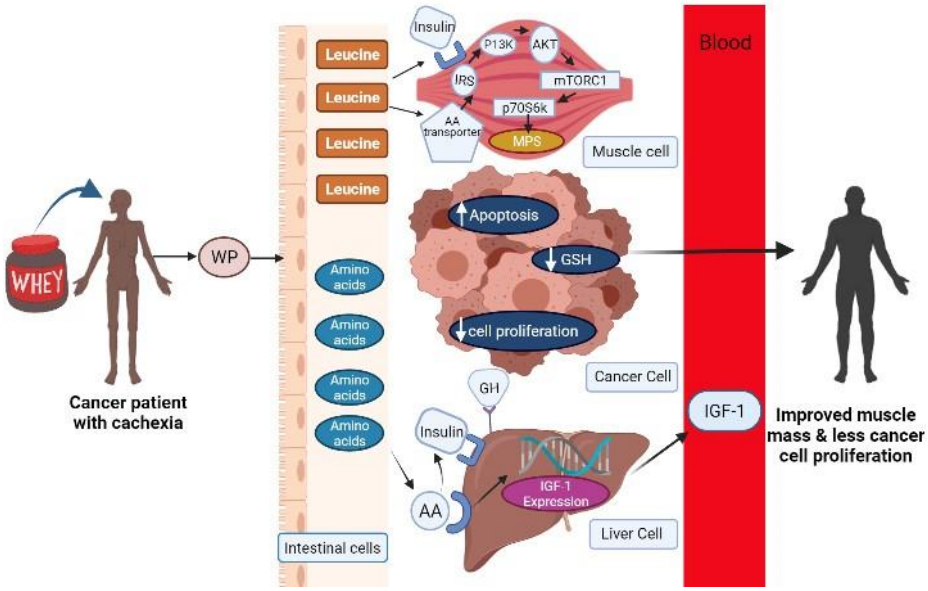


Figure 6. WPs' mode of function in cancer

Source: A. Thampy et al. 2024. The effectiveness of whey proteins in prevention and treatment of cancer: a review, Critical Reviews in Food Science and Nutrition. p- 2088-2104 (11)

2.3 Tomatoes

2.3.1 Role of tomatoes in cancer

The dietary and therapeutic effects of tomatoes (*Lycopersicon esculentum*) are magnificent. They work as a natural reservoir of a variety of antioxidant chemicals that are valuable for health. Gautam et al. 2016 reported that tomatoes had high antimutagenic activity in various *in vitro* models (68). Gupta et al. 2015 revealed that tomatoes were the largest contributor to lycopene intake in humans (69). They also pointed out the multifunctional role of lycopene as a "nonsurgical aid in the treatment of oral diseases, including OSCC." Lycopene's anticancer properties have been proven in both *in vitro* and *in vivo* tumour models. The processes underlying lycopene's inhibitory effects on carcinogenesis were explained by the mechanisms of "reactive oxygen species (ROS) scavenging, upregulation of detoxification systems, interference with cell proliferation, induction of gap junctional communication, inhibition of cell cycle progression and arresting cell cycle in different phases, increase induction of apoptosis, etc" (26,69) (Fig. 7). It was also found to raise the amounts of the p53 protein in cancer cells, arrest the cell cycle via modulating cell cycle regulatory proteins, and alter mitochondrial function. More than that, the Ras-dependent activation of NF-κB was reduced by lycopene, which was accompanied by an inhibition of ROS production and a decrease in the phosphorylation of JNK, ERK1/2, and p38. And lycopene suppressed Akt activation and regulated downstream targeted molecules such as cyclin D1 and p27 to inhibit cell proliferation (26). Moreover, lycopene was recognised as safe for daily dietary intake with nontoxicity (69). According to a comprehensive study by Singh and Goyal 2008, lycopene in tomatoes has been shown to be protective against a number of malignancies in humans, including colorectal, prostate, breast, lung, and pancreatic cancers (70).

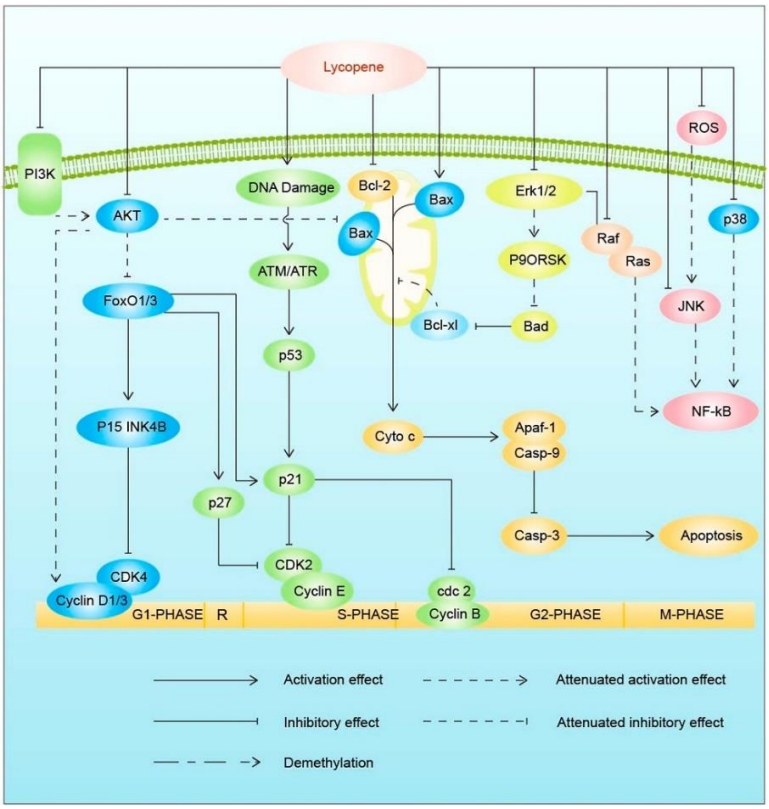


Figure 7. Lycopene's mode of function in cancer

Source: Jia et al. 2021. Investigating into anti-cancer potential of lycopene: Molecular targets, Biomedicine & Pharmacotherapy. p- 1-11 (26)

2.4 Banana

2.4.1 Role of banana in cancer

Bananas are packed with functional, health-promoting nutrients such as Vit C, provitamin A carotenoids, potassium, and fibre (27). They are undoubtedly a healthy fruit choice due to their nutritional profile. It is true that bananas are sometimes referred to as "poor man's apple," since they are readily available and less expensive, making them a healthy option, affordable for everyone (71). It possesses an unusual blend of energy value, vitamins, minerals, protein, and tissue-building components. Because it has less water and more solids than other fresh fruits, it is also a good source of calories. The role of bananas in relation to cancer has been a topic of interest in nutritional research. While bananas are a healthy fruit rich in various nutrients,

studies have explored potential connections between banana components and their influence on cancer.

The *Nendran* variety banana (*Musa* AAB group) is a multipurpose fruit that can be eaten raw as a fruit or in prepared form (28). It is a sterile triploid that is cultivated in warm climates for its tasty yellow-skinned fruits. It is commonly called edible banana or French plantain. The fruit pulp is abundant in minerals like potassium, magnesium, and calcium, as well as Vit C, dopamine, phenols, flavonoids, and carotenoids (βC) along with antioxidant enzymes such as ascorbate peroxidase, catalase, peroxidase, and superoxide dismutase, all of which have significant implications in the prevention of cancer (28,29). According to Dhandapani et al. 2017, the edible part of the *Nendran* (AAB) has the greatest βC content amongst the major Indian banana varieties that has functional anticancer properties (30). Antioxidants help neutralize free radicals in the body, which are known to cause cellular damage that may contribute to cancer development. Its fruit peel has shown potent cytotoxic activity against MCF-7 breast cancer cell lines.

2.5 Flaxseeds and sesame seeds

2.5.1 Role of flaxseed and its oil cakes in cancer

Flaxseeds (*Linum usitatissimum*) and sesame (*Sesamum indicum*) seeds have an array of therapeutic dietary uses that offer potential health advantages. Both are excellent carriers of lignans, which is an oestrogen derived from plants. By attaching to oestrogen receptors on breast and endometrial tissue cells, lignans have the potential to function as a mild oestrogen (72). This may shield cells from the damaging effects of oestrogen exposure that can lead to cancer. Following the oil extraction process from oilseeds, a byproduct remains, commonly referred to as “oilseed meal or oilseed cake.” These remnants serve as livestock feed or are disposed of as refuse to a large extent earlier. Sesame seed oil cakes (SOC) and flaxseed oil

cakes (FOC), byproducts of sesame as well as flaxseed oil production, have emerged as a potential source of health benefits.

Numerous investigations have been conducted to ascertain the potential involvement of flaxseed phytoestrogens (plant-based chemicals that resemble oestrogen) in preventing cancer. Consuming flaxseeds has been demonstrated to impair receptors for oestrogen on breast tissue cells in tests on animals (72). This has been suggested as a possible method to halt the growth of tumours and shield cells from disruption. Lignans, a phytoestrogen, and alpha-linolenic acid (ALA), two flaxseed constituents, have been investigated in connection with cancer. Secoisolariciresinol Diglucoside (SDG) is one of the main lignans prevalent in flaxseed products. Rodriguez-Leyva et al. 2010 identified flaxseed as one of the richest sources of the plant-based omega-3 fatty acid- ALA, which has been shown to downregulate cell proliferation of many cancer cells (73). According to this study, 1 tbsp flaxseed oil contains about 7.249 g of ALA. Reethega et al. 2018 proved the cytotoxicity of flaxseed oil on the KB OC cell line and established that it can be used for OC patients (74). Sylva and Alcorn 2019 studied the protective effects of flax lignans in carcinogenesis with respect to modulation of cell signalling and metabolism, cell growth and differentiation, cell motility and cytoskeletal dynamics, cell cycle, angiogenesis, and apoptosis (32).

Another powerhouse of protein and fiber is the FOC, which can be employed for people to consume in plenty of ways. It has up to 36.5-38.5% protein, 2–7% fiber, and is high in vitamin B6, which functions as a coenzyme in enzymatic activities (31). It also boasts a considerable total phenolic content (TPC), ranging from 776 to 2255 mg per 100 grams, with variations based on the extraction solvent used. Additionally, FOC is also rich in polyunsaturated fatty acids (PUFAs), particularly ALA (omega-3) and conjugated linolenic acid, offering a myriad of health benefits, including maintaining low levels of low-density lipoprotein (LDL), along with exhibiting anticancer, antihypertensive, antidepression, antiaging, and antiarthritis effects

(31). Besides, lignans found in FOC have been associated with a reduced risk of certain cancers, including breast, prostate, and colon cancer, due to their antioxidant, anticarcinogenic, antimutagenic, anti-proliferative, antiangiogenic, anti-invasive, antimigratory, and antiestrogenic properties, along with induction of cell death (32). In addition, there are several indications showing that eating foods high in dietary fiber lowers the risk of colon cancer. Increased intake of foods and dietary supplements high in omega-3 fatty acids may also reduce the risk of colorectal and breast cancers, according to some research (75).

2.5.2 Role of sesame seed and its oil cakes in cancer

Sesame seeds are loaded with carbs, crude fiber, oil, and particularly, protein. Sesame protein is perhaps the most nutrient-dense of all the oilseed proteins, primarily because it encompasses a lot of tryptophan and sulphur-containing methionine amino acids (76). The "Queen of oilseeds" is so named due to its larger and varied applications (76). According to Johnson et al. 1979 and Daghir et al. 1967, sesame protein has a high methionine content, which is uncommon for most plant proteins. Additionally, the defatted meal made from dehulled seeds is free of unwanted colours. In a study by Wu Ming-Shun et al. 2019, sesame lignans were found to manifest anticancer effects against tumour cells of many cancers in *in vitro* and *in vivo* studies (77). Kumar and Singh 2015, identified sesamol as the best antioxidant and free radical scavenger lignan in sesame oil, and reported that sesame oil can be used as a stabilizer for prolonging the shelf life of oils and oil-based products (78). *In vitro* studies in rats by Liu et al. 2006, reported that sesame seeds are the rich source of mammalian lignan precursors (sesamin) (79).

SOC is also a protein-rich byproduct that varies in protein content depending on the extraction method. It typically contains around 35-38% protein, fat content ranging from 5 to 20%, and fiber content ranging from 4-12%, with approximately 2% lignan (31). It is a protein-packed, nutritious byproduct with a distinctive amino acid profile, that is low in lysine but abundant in

sulphur-containing amino acids. SOC also provides good protein digestibility, accounting for around 78%. The majority of its fatty acids are unsaturated, primarily oleic acid (C16:0) and linoleic acid (C18:1), making up 80% of total fatty acids (31), contributing potential health benefits.

2.6 Emerging trend of whey-based products

Being one of the quickly emerging economies, the Indian food industry has experienced a tripling in growth over the past decade. This upward trajectory is anticipated to continue for the next decade. Natural products are relatively safe for human consumption, which has boosted their popularity across the food industry. While their demand in the food and beverage industries grows by the day, the breadth of functional foods and beverages expands as well. Functional foods and beverages have acquired immense popularity among the health-conscious Indian population. Energy drinks and different types of RTS beverages are picking up steam in the functional beverage market, offering tremendous convenience and portability to today's busy customers. As a result, substances that are naturally present have been deemed exciting, key capitals in the realm of pharmacotherapy that aid in the treatment of numerous non-communicable disorders, and they have acquired enormous acclaim within the health-conscious Indian populace (10).

This upsurge within the nation is a significant catalyst in the expansion of the market, as WP is an integral constituent in functional goods such as RTS beverages or meals and nutritious snacking options, among others. The Indian WP market is primarily fuelled by multiple variables, such as the growing appeal for milk-based products, exacerbated public understanding regarding the importance of maintaining a healthy diet, the growing enthusiasm of youngsters in athletic and other sports endeavours, and the subsequent growing popularity of fitness centres and health clubs to help the public stay fit. Besides, WP, with its role in muscle building and repair, is perhaps the type of protein that attracts the most attention among

fitness enthusiasts and has held a prominent position among protein powders in the sports nutrition sector for ages (19). The progression onto the heightened processing of whey into whey derivatives, including whey protein isolates (WPIs) and whey protein concentrates (WPCs), has garnered traction (49).

Since the beginning of WB production in the 1970s, an extensive variety of WBs have been developed to the current time (80). Apart from being an outcome of cheese production, whey is now utilized in the creation of a broad range of meals and additives, comprising pharmaceutical items, food supplements, fermented foods, beverages, semisolids, and so on. Fruit and vegetable-based WBs are gaining tremendous focus due to their expanding market potential. Although a wide range of WBs, including plain, carbonated, alcoholic, and fruit and vegetable juice additions (81), have been fruitfully produced and distributed worldwide, the majority of these products are primarily intended to quench thirst, and the therapeutic possibilities of enrichment with other phytochemical-rich dietary components have not been fully explored and are poorly documented. Due to its characteristics, functions, and chemical structure, whey is an ideal compound to replace more conventional substances or a fantastic foundation for the development of a variety of novel items.

2.6.1 Utilization of whey in beverages

The historical utilization of cheese whey as a beverage, particularly for curative intentions, appears to be brought far back to ancient Greece. The ancient Greeks were the first to consume cheese whey as a drink, due to therapeutic reasons; in 460 B.C., Hippocrates endorsed whey to treat a variety of human maladies (82). The Indian dairy setting is characterized by the production of whey, a byproduct that is a significant aspect of the country's extensive cheese manufacturing (49). Today, WBs encompass a diverse array of food items that emerge through the combination of acid, native, or sweet whey, deproteinized whey, and powdered whey with various components. These components may include tropical and other fruits and vegetables,

agricultural products (primarily bran), vegetable protein isolates, cocoa, and aromatizing extracts such as vanilla extracts (83). A broad range of WBs, comprising plain, carbonated, alcoholic, fruit, and vegetable juice additions, have been fruitfully produced and distributed worldwide because they hold excellent prospects for utilizing whey solids (81). Dietary beverages, beverages containing hydrolysed lactose, milk-like beverages, and powder drinks are also classified as whey beverages. The most prevalent variety of novel whey beverages are fruit juice and whey blends. The primary benefits linked to these beverages are the nutritional amalgamation of the fruit foundation, vitamin-rich constituents, and whey and calcium proteins derived from dairy. Due to the relative ease of producing whey-fruit juice mixtures, numerous dairy processors have endeavoured to penetrate the beverage industry by developing their own variations on the common theme.

The global market for whey ingredients is undergoing significant growth, particularly in the context of opportunities related to infant formulas, nutritional foods, and medical applications (49). At present, the market offers an assortment of WBs, such as those fortified with WPs, deproteinated WBs, flavoured and fermented dairy-based drinks that incorporate whey or its elements, etc. Certain items may also be viable candidates for marketing in their desiccated state. Given the rising consumer interest in fruit infusions and cold beverages, whey exhibits considerable potential as a component in beverage formulations. It can replenish a significant amount of lost organic and inorganic salts to the extracellular fluid when consumed (51). Additionally, it contains nearly all of the electrolytes found in oral rehydration solution (ORS), which is consistently employed to regulate dehydration.

Figure 8 below represents the classification of different whey beverages, and Tables 3 and 4 denote different types of WB formulations and other whey-based products, respectively.

Classification of WBs

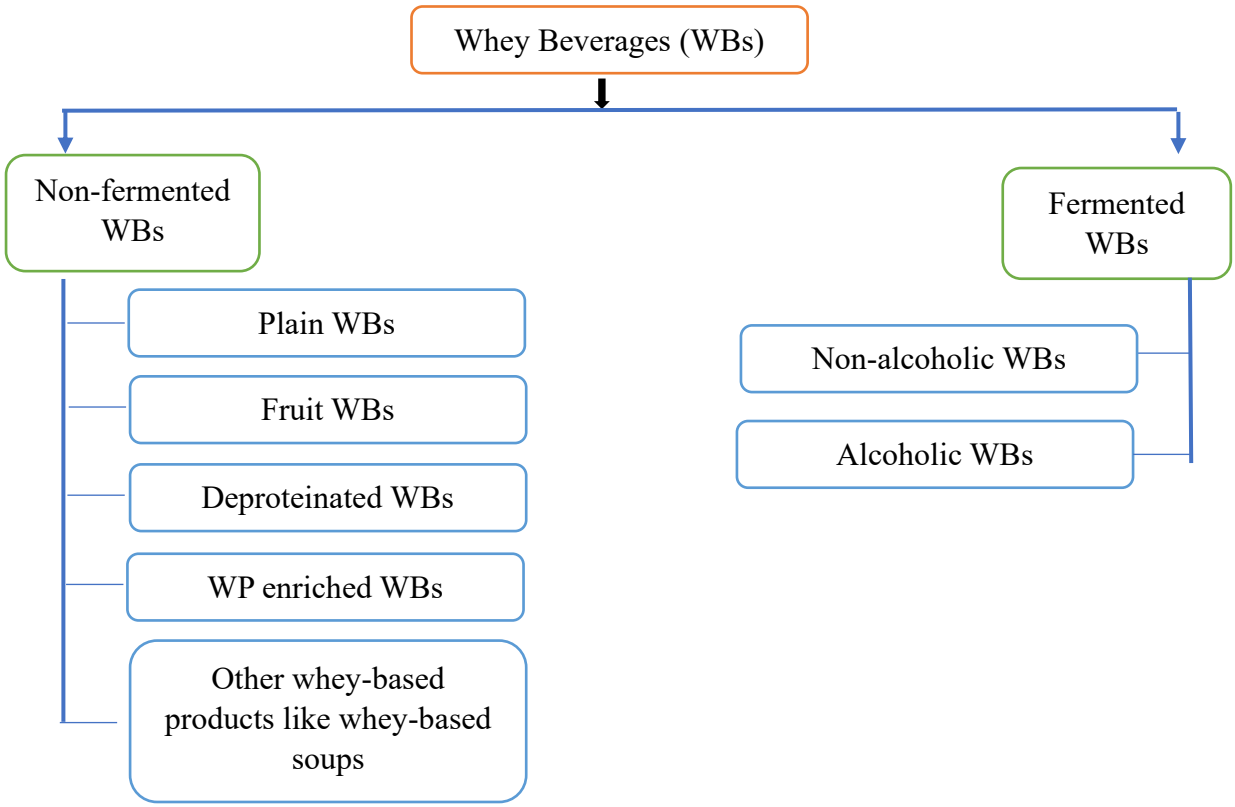


Figure 8. Classification of WBs

Source: Holsinger et al. 1974. Whey Beverages: A Review, Journal of Dairy Science. p- 849-859 (82)

Table 3. Different types of WB formulations

WB type	Major outcomes	References
Whey-mango mixed beverage	There was a considerable increase in TSS, acidity, reducing sugars, moisture, fat, carbohydrate, and protein in prepared beverage samples after 25 days of storage. There was merely a 1% sedimentation rate. The produced samples showed an acceptable overall plate count, mold count, and yeast count, with the number of coliforms less than the detection limit of <1.	Ahmed et al. 2023 (84)
Whey orange beverage (WOB)	The "microbiological, physicochemical, and functional quality studies" of WOB processed by microfiltration (0.2 µm) exhibited better "preservation of bioactive compounds and related functional activities, with similar microbial quality and rheological parameters compared to conventional heat treatment when used with a lower feed temperature of 20°C - 30°C."	Vieira et al. 2020 (85)

Fermented whey tomato beverage	The pectin-carboxy methyl cellulose (P-CMC)-added fermented beverages were stored for 12 days at ±4°C, shown that the P-CMC combination decreased "the rate of lactic acid rise, total lactic acid bacteria, antioxidant activity, and pH during the course of 12 days of storage." The optimal combination of "cheese whey and tomato juice to make a fermented beverage is 0.1%:0.3% (w/v) addition of P-CMC, which provides a 34.636% hypocholesterolemic impact."	Saidah et al. 2019 (86)
Whey guava beverage (WGB)	The studies on physicochemical, microbiological and sensory properties of WGB concluded a gradual increase in acidity with a decrease in Vit C, lactose and calcium content, total bacterial counts, yeast and mold count with increasing storage period for 3 months. The blend with 60% whey and 40% guava pulp reported highest sensory score. °C	Moussa and Manal 2019
Whey pineapple beverage (WPB)	The WPB with 30:70 ratio of whey and pineapple juice sterilized at 85 for 15 min was found better in almost all physicochemical, microbial and sensory quality parameters with a storage life of 20 days as compared to other combinations.	Pandey et al. 2019
Whey kiwi beverage (WKB)	An investigation on WKB made from chhana whey resulted in an increased fat, protein, total sugar, total solids, titratable acidity, pH, ash and total fiber content in kiwi pulp added samples. WKB with 9% kiwi pulp and 8% sugar gave desirable nutritional profile.	Hande and Chavan 2019
Whey watermelon beverage (WWB), Whey passion fruit beverage (WPFB) and Whey Indian gooseberry beverage (WIGB)	Among the three different combinations of whey RTS beverages, WPFB was more sensorially and nutritionally accepted compared to other formulations.	Gimhani and Liyanage 2019
Fermented whey beetroot beverage (FWBB)	Using Kluyveromyces lactis strains, a probiotic-potential antioxidant-rich FWBB was synthesized, with viability above 75% following exposure to simulated stomach and duodenal fluids.	Oliveira et al. 2019
Electrolyte whey drink (EWD)	Rehydrating EWD was effectively prepared from paneer and cheese whey by enzymatic hydrolysis of lactose and salt (sodium, potassium and citrate) level adjustment to make it to 245 mOsm/L ORS requirement as per WHO.	Pushpa, Kempanna, and Murthy 2018
Whey guava beverage (WGB)	A comparative study of WGB processed by cold plasma technology (CPT) and pasteurisation provided greater preservation of fatty acid profile,	Silveira et al. 2018

	bioactive and volatile compounds. Changes in fatty acid profile and ACE inhibitory activity was dependent on time duration and flow rate used for plasma operation.	
Whey probiotic orange RTS beverage (WPOB)	The final optimised beverage had a ratio of 60:40 orange juice and whey probioticated at 2% with a keeping quality of 28 days at refrigerator maintaining highest viable count of probiotics without addition of any preservatives.	Thakkar et al. 2018
Whey pineapple beverage (WPB)	The formulation of WPB with 35% pineapple juice and 65% whey scored highest for overall acceptability.	Munasinghe and Dilrukshi 2018
Whey mango beverage (WMB)	A mango flavoured sweetened whey drink was prepared by using whey, sugar, and mango pulp. Mango pulp added treatment scored higher value by the sensory panel than the control which also increased the deliciousness and nutritive value of the drink.	Karthikeyan 2018
Whey soybean chocolate beverage (WSCB)	A mixed drink of WSCB was developed and tested by a simplex-centroid design and RSM was used. The results indicated a higher sensory impression and purchase intention with higher desirability index with thickeners.	Oliveira, Granato, and Barana 2018
Whey grape juice beverage (WGJB)	The physical and sensory property analysis of WGJB processed by supercritical carbon dioxide technology (SCCDT) reported that SCCDT can be used as an alternative for WGJB processing.	Amaral et al. 2018
Whey pineapple beverage (WPB) and Whey orange beverage (WOB)	The WPB with 30% pineapple juice and WOB with 25% orange juice displayed most sensorially accepted beverage blend with a shelf life of about 12-16 days for optimized WPB.	Prashanth et al. 2018
Whey probiotic mango beverage (WPMB)	The paneer WPMB with 15% mango juice, 10% sugar level and 3% probiotics were found to have higher acceptability scores and this optimized WPMB exhibited a keeping quality of 3 days at ambient temperature and up to 21 days at refrigerated temperature against control sample.	Lakshmikanth, Puranik, and Soumya Shree 2018
Whey watermelon beverage (WWB)	The formulated WWB with 10% of juice received highest acceptability and also increased the deliciousness and nutritive value of WWB.	Punnagaiarasi, Elango, and Karthikeyan 2017
Whey beetroot beverage (WBB)	The blend consisting 80% paneer whey, 20% beetroot extract with 6% menthol extract and 7% sugar was shown to be significantly superior to other treatments in terms of organoleptic qualities with a score of 8.51 in a 9-point hedonic scale. Completely Randomized Block Design (CRBD) was used to evaluate the data.	Satpute et al. 2017

Whey orange herbal beverage (WOHB)	The WOHB with 74% whey, 20ml orange juice, 6ml each of basil, mint, ginger, alovera and lemon grass extracts with stevia scored highest in sensory hedonic scale.	Maya, Dubey, and Ritu 2016
Whey strawberry flavoured beverage (WSFB)	A comparative sensory evaluation study of strawberry flavoured whey and yogurt sold in market found that products with higher levels of brightness, artificial strawberry taste and aroma, sweet taste; intermediate smoothness of mouthcoating, colour, and viscosity; and low particles, acid taste, and aroma were more preferable.	Janiaski et al. 2016
Whey flaxseed oil beverage (WFOB)	A functional beverage from whey enriched with flaxseed oil (0.2% cold-pressed) was formulated with a favourable ratio of n-6/n-3 PUFA and low atherogenic and thrombogenic indices.	Kabasinskiene et al. 2015
Whey orange beverage (WOB)	The optimised formulation of WOB had a ratio of 3:2 whey and orange juice with 8% sugar (w/v) and 0.1% stabilizer (w/v). It had a shelf life of 11 days at room temp and up to 3 months at refrigerated temp with addition of preservative.	Chatterjee et al. 2015
Whey tomato RTS beverage (WTRB)	The best treatment of 65% paneer whey and 35% tomato juice with standard ingredients of RTS beverage was found to have enhanced shelf life by the addition of 100 ppm sodium benzoate with a decrease in sensory score, pH, ascorbic acid, total sugar and lycopene content and concomitant increase in acidity, TSS, and reducing sugar content upon storage.	Bangaraiah et al. 2014
Whey based fructooligosaccharide drink (WFOSD)	Compositional analysis of WFOSD showed that addition of FOS as a prebiotic soluble fiber enhanced the functional properties of whey.	Yasmin et al. 2014
Whey carrot beverage (WCB)	The prepared WCB blended with grape or sesame oil was rich in proteins, minerals, β-carotene, antioxidants and PUFA with improved functionality.	Raoaf, Nasr, and Mostafa 2014
Whey guava beverage (WGB)	Different treatments of WGB were analysed at various processing time temperature for sensory, chemical and microbial parameters for 90 days. The WGB with 67.5% whey and 20% guava pulp pasteurized for 65°C for 25 mins scored top in sensory quality after 45 days with better pH, acidity, protein, total sugar and reducing sugar content than other samples with respect to shelf life and microbial studies also.	D. Singh, R. Singh, and Bhatt 2014
Whey barley multi-ingredient beverage (WBMB)	Ingredients used for developing WBMB were whey water (70 ml), Barley water (30 ml), Sugar (10g), green tea (5ml), Premix (90mg), FOS (2g),	Jain, Gupta, and Jain 2013

	Glucosamine sulphate (1 tab), Flax seed powder (5ml), Digene (1/3rd of tab), and Sodium citrate. The fortification of whey with these ingredients elevated its functional properties and resulted in an economic viability of whey utilization.	
Whey prickly pear beverage (WPPB)	Physical stability study of WPPB done by factorial-design matrix with whey treatment, sugar (S) and pectin (P) level being the factors tested showed that it was physically stabilized by S and P amount increase using the heat-treated whey.	Baccouche et al. 2013
Whey orange beverage (WOB)	A comparative study of orange beverage (OB) and WOB showed that WOB contained higher levels of protein, ash, vitamin B2 and lower levels of sucrose, Vit C and antioxidant activity than OB with overall comparable sensory profile with that of OB.	Sady et al. 2013
Whey mango beverage (WMB)	The developed WMB using whey, mango powder, flaxseed oil and stabilizer was rich in polyphenol and carotenoids and exhibited high antioxidant capacities.	Gad et al. 2013
Whey strawberry flavoured probiotic beverage (WSPB)	Various mathematical models employed to optimize whey content in WSPB showed that 49% and 65% of cheese whey can be used effectively for probiotic beverage formulation.	Castro et al. 2013
Whey pineapple bottle gourd mixed herbal beverage (WPBHB)	The preparation of WPBHB with 2% mint leaves extract presented a shelf life of 15 days acceptability with improved nutritional and organoleptic profile in refrigeration temperature without adding chemical preservative.	Baljeet, Ritika, and Sarita 2013
Fermented dairy beverage (FDB)	Fermented dairy beverages made with goat's milk, cow's milk and a mixture of the two milks along with whey and guava jelly was affected by milk type and storage time without changing much of the sensory profile and suggested the replacement of stabilizer by use of fruits jellies.	Gomes et al. 2013
Whey lemon beverage (WLB)	The preparation and optimization of WLB by the use of binary sweetener blend aspartame ⁄saccharin (0.0425%), was found to be effective and scored highest as compared to single sweeteners aspartame (0.07%) and saccharin (0.045%) with nonsignificant differences with the control WLB sweetened with sucrose in all sensory attributes.	Meena et al. 2012
Whey tomato juice beverage (WTJB)	The developed WTJB was optimized by RSM engaging Central Composite Rotatable Design (CCRD) with an overall acceptability of 8.5 on 9-point hedonic scale (9PHS).	Rajoria, Chauhan, and Kumar 2011

Table 4. Different types of other whey-based products

Whey based Products	Major findings	Reference
Whey permeate mixed kefir like fermented beverage (WPKLB)	Fortification of a carob-based kefir-like beverage (KLB) with whey permeate (WP) and oat flour (OF) was optimized using WP at 11.51% and OF at 4.77% by RSM engaging CCRD with enriched bioavailable phenolic derivatives and highly digestible proteins.	Sana et al. 2021
Whey pectin jelly	Physicochemical and microbial analysis of paneer whey pectin jelly was carried out and recorded no counts of yeasts and moulds.	Wasnik 2016
Kefir whey date beverage (KWDB)	Using RSM engaging CCRD, the optimal process parameters identified for KWDB were 36.76% (w/v) date syrup, 2.99% whey permeates, and 2.08% kefir grains inoculum size and aided to develop a product with acceptable organoleptic qualities as well as significant antioxidant activity.	Sana et al. 2019
WPS enriched with microencapsulated PUFA vegetable oil	WPS with walnut and green coffee oil-loaded microparticles resulted in an efficient protection of the oils against oxidative degradation, unaffected flow behaviour or viscosity. Sensory profile was not influenced for walnut oil but declined for green coffee oil at 7.5% concentration.	Rojas et al. 2020
Rice cooked in paneer and cheese whey (PWR and CWR)	Sensory evaluation and rheological studies of PWR and CWR presented a higher overall acceptability for PWR compared to control and CWR.	Chaudhary and Balasubramanyam 2015
WPC and fruit juice enriched misti dahi	Misti dahi with 2% WPC and apple and orange juice at 15% level scored top in sensory quality with improved functional properties such as water binding capacity, gelation, viscosity and emulsification of the product.	Soumya Shree et al. 2017
HWP fortified mango RTS beverage	The mango RTS beverage fortified with 3% HWP was found acceptable with good sensory appeal and stability during thermal processing as well storage in glass bottles without any sedimentation when compared with native whey protein fortification.	Yadav et al. 2016
WPC enriched tomato beverage	The top reconstituted beverage optimized using RSM engaging CCRD was comprised of WPC 4.98g, sugar 15.71g, Guar gum 0.93g and 100g tomato juice concentrate.	Rajoria, Chauhan, and Kumar 2015

2.6.2 WBs in the Indian market

The ready-to-drink (RTD) WB market in India is still developing, but there are a few brands actively marketing their products in the Indian market, such as Hatsun Agro Product Limited, Tamil Nadu Dairy Cooperative Federation (TCMPF), Amul, etc. TCMPF is marketing a whey-based drink, namely Aavin whey drink, which is made with ingredients of fruit pulp or extract, sugar, and whey, along with citric acid and stabilizer (INS 462/469) according to variant. Three different variants of pineapple, mango, and jaljira whey drinks are manufactured by them with a storage period of seven days under 5ºC temperature. Another RTD WB called Aniva whey drink is available in multiple flavours (mango, lemon, and orange) and is sold by Hatsun Agro, product limited, a dairy company based in Tamil Nadu, India. “Amul pro,” a malt-based, WP-added powder from the popular house of Amul, is getting popular in the Indian beverage market, which is consumed by adding to milk. Ingredients consist of milk, whey and cocoa solids, sugar, along with malt extract, DHA, salt, and other additives, and are shelf stable up to one year. In addition, Amul provides a sports drink known as "Amul Stamina" that contains WP, other milk solids, sugar, and other ingredients to promote muscle growth and replenish the body's fluids. The acidophilus soft drink known as "Acidowhey" and "Whevit" soft drink was formulated at the National Dairy Research Institute, Karnal, using whey. "Acidowhey" is a carbonated beverage that undergoes fermentation using a specific strain of lactic acid bacteria while preserving the complete nutritional value of whey. Both brands highlight the convenience aspect of RTD beverages compared to mixing WP powder. Several options for WP brands are emerging now, catering to the needs of health-conscious consumers. Popular WP brands available in the Indian market are “Optimum Nutrition, Ultimate Nutrition, MuscleBlaze, Dymatize, AS-IT-IS Nutrition, Patanjali Nutrela Whey Performance Protein,” etc. These brands offer a variety of WP powders, protein bars, ready-to-drink shakes, and other whey-based supplements, providing options for different tastes, dietary requirements, and fitness goals.

CHAPTER 3
AIM & OBJECTIVES

3.0 AIM AND OBJECTIVES

3.1 AIM

- To evaluate the anticancer potential of a whey-based multi-ingredient beverage

3.2 OBJECTIVES

1) To formulate and optimize a whey-based multi-ingredient beverage
2) To determine the sensory profile, physicochemical properties, shelf-life studies, nutrient composition analysis, total antioxidant capacity, and total phenolic content of the developed whey-based multi-ingredient beverage
3) To evaluate the anti-cancer potential of the developed whey-based multi-ingredient beverage through in vitro studies using the KB oral cancer cell line

3.3 RESEARCH QUESTION

- Does whey based multi-ingredient beverage exhibit anticancer properties in oral cancer cell line?

CHAPTER 4
RESEARCH METHODOLOGY

4.0 RESEARCH METHODOLOGY

The materials used and the methodology adopted for the study have been discussed under the following sub headings:

4.1 Materials

4.1.1 Ingredients

4.1.2 Containers

4.1.3 Cell lines

4.1.4 Chemicals

4.1.5 Apparatus/Instruments

4.2 Methods

4.2.1 Study area

4.2.2 Procurement of ingredients

4.2.3 Procurement of cell lines and chemicals

4.2.4 Quality analysis of ingredients

4.2.5 Processing of ingredients

4.2.6 Formulation and optimization of WMB

4.2.7 Formulation of WMB mixes

4.2.8 Quality analyses of WMB mixes

4.2.9 Anticancer activity assays of T1 WMB mix

4.2.10 Statistical analysis

4.1 Materials

4.1.1 Ingredients

Various ingredients used for the formulation of WMBs are whey, tomato, banana, flaxseeds, and sesame seeds (Fig. 9). These ingredients have been selected for their potential health benefits, particularly their antioxidant and anticancer properties. The details of the selected ingredients are given below in Table 5.

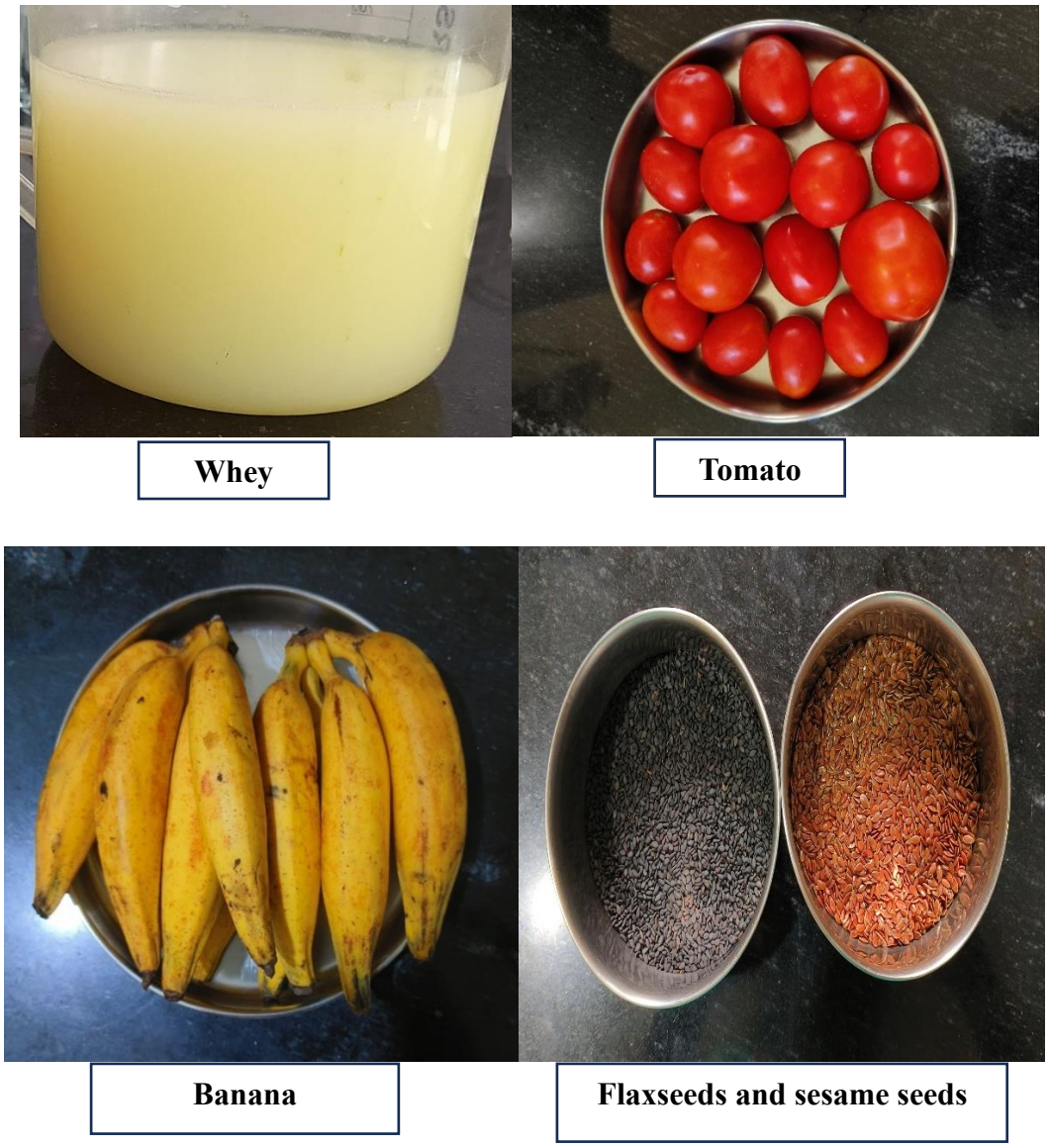


Figure 9. Various ingredients used for the formulation of WMBs

Table 5. Details of selected ingredients for WMB formulation

Sl. No.	Ingredient	Variety, if any	Rationale for selection of ingredients
1.	Whey	Cheese whey	**1.** Kolar is famous for "KOMUL" and large quantities of cheese whey obtained during cheese making is disposed as waste. This disposal is a huge environmental trouble due to its high BOD (22) **2.** Excellent nutritional profile and anticancer properties (11,15) **3.** As a means of whey valorization
2.	Tomato	Arka Apeksha	**1.** F_1 hybrid developed by IIHR, Bangalore, suitable for both processing and fresh market (126) **2.** Triple disease resistance power **3.** Good TSS (4.0-4.7° Brix) and colour value with high lycopene content of 11 mg/100g (127) **4.** Very common and easy to get **5.** Lycopene, the primary pigment found in tomatoes, has the highest level of antioxidant activity and it also exert significant anticancer properties (26)
3.	Banana	Nendran banana	**1.** Highest βC content among Indian bananas (30) **2.** High TSS content (and carbohydrate), suitable for processing (128) **3.** Rich in Vit C, potassium, magnesium, calcium, fibre and phytochemicals such as provitamin A carotenoids (βC, unlike other banana), phenols, tannins, flavonoids, and flavanoids which exhibits anticancer effects (27,28,71) **4.** Also rich in antioxidant enzymes such as ascorbate peroxidase, catalase, peroxidase and superoxide dismutase all of which have significant implications in the prevention of cancer (28)
4.	FOC	Brown coloured	**1.** High protein, fiber, polyphenols (lignans), TPC and ALA content along with good protein digestibility (31) **2.** FOC lignans and ALA - Reduced the risk of certain cancers due to their antioxidant, anticarcinogenic, and anti-proliferative properties (31,32)

			3. Large quantities of FOC obtained is disposed as refuse or used as livestock feed and is not fully utilized for human consumption, hence as a means of FOC valorization
5.	SOC	Black coloured	**1.** High protein (the most nutrient-dense protein of all the oilseed proteins due to high tryptophan and methionine), fiber, polyphenols (lignans), and unsaturated fatty acids content along with good protein digestibility (31,76) **2.** Polyphenols (lignans) were found to manifest anticancer effects against tumour cells of many cancers (77) **3.** Large quantities of SOC is also disposed as refuse or used as livestock feed and is not fully utilized for human consumption, hence as a means of SOC valorization

4.1.2 Containers

Various containers including collection and storage containers comprising steel vessels, cold storage box, measuring cups, muslin cloth, crucibles, desiccator, and as well as packaging materials of low-density polyethylene (LDPE) films (70 µm thickness), metallized polyethylene (MPE) films and glass bottles were used for the shelf-life studies.

4.1.3 Cell lines

Two cell lines namely, KB OC cell line and 3T3-L1 cell line, which is a murine fibroblast cell, as non-tumoral lineages were used for the study to evaluate and compare the cytotoxic potential of developed WMB mix.

4.1.4 Chemicals

Various chemicals, reagents, diagnostic kits and standards used in the study is briefed in this section. All the chemicals, including HPLC-grade methanol, ethanol, acetone, hexane, petroleum ether, ascorbic acid, gallic acid, quercetin, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), acetic acid, sodium carbonate, hydrochloric acid (HCL), nitric acid (HNO_3), sodium citrate buffer, Folin–Ciocalteu's reagent, aluminium

chloride, ammonium molybdate, potassium acetate, sulfuric acid (H2SO4), sodium phosphate, metaphosphoric acid, ethylene diamine tetra acetic acid (EDTA), sodium dihydrogen orthophosphate (NaH2PO4), ortho-phthalaldehyde (OPA), amino acids standards, multielement standard, were obtained from Sigma Aldrich and Hi-media Chemicals (Bangalore, India). All other chemicals and reagents used were of analytical grade, and milli-Q water was used for HPLC analyses. All the tests were performed according to the manufacturer's guidelines.

4.1.5 Apparatus/Instruments

Various apparatus/Instruments used in the study are given below in Table 6.

Table 6. Details of apparatus/instruments used

Sl.No.	Equipment	Purpose	Make/Model
1	Cabinet drier	To dry tomato and banana	-
2	Electronic balance	To weigh the chemicals and samples for analysis	Contech
3	Weighing balance	To weigh samples	-
4	pH meter	To measure the pH of samples	Agilent Technologies 3200P pH meter
5	Water activity meter	To measure the water activity of the samples	Novasina Lab Touch-aw, Switzerland
6	Viscometer	To measure the viscosity of samples	Brookfield DV-II+Pro, USA
7	Hot air oven	To dry the samples	(Heraeus)
8	Fruit mill	To crush the fruits	Bajaj Processpack Limited, India
9	Steam jacketted kettles	To heat the crushed fruits	Bajaj Processpack Limited, India
10	Fruit pulper	To extract pulp from crushed fruits	Bajaj Processpack Limited, India
11	Electric hot plate	To boil samples	Bajaj Vacco, India
12	Cold press oil extraction machine	To extract oil from oil seeds	-
13	Muffle furnace	To measure the ash content in the samples	Scientek Hub, India

14	Elemental analyser	To estimate the nitrogen in the samples	Thermo Scientific Flash 2000 Organic elemental analyser, Italy
15	Mixer grinder	To make fine powder of the drum dried sample flakes	Panasonic MX-AC555 1000-Watt, India
16	ICP-AES	To estimate the mineral content in the samples	ACTIVA-M, Horiba Jobin-yvon, USA
17	Moisture analyser	To estimate the moisture content	Denver Instrument IR 35, Germany
18	Soxhlet apparatus	To estimate the fat	-
19	Drum dryer	To drum dry the samples	-
20	SEM	To assess the morphological structures of samples	ZEISS, Germany
21	Fluorescence microscope	To assess the morphological changes of cells	-
22	Magnetic stirrer	To uniform the sample before analysis of chemical parameters	Tarsons, SPINOT,
23	Centrifuge	To collect the supernatants for analysis	R8C, Remi, India
24	Spectrophotometer	To measure the optical density of components	Microplate reader, Thermo Scientific evolution 220, CA, USA
25	HPLC	To estimate Vit C content	Nexera HPLC, Shimadzu Corporation, Japan
26	UHPLC	To identify amino acids	Nexera UHPLC, Shimadzu Corporation, Japan
27	GC-MS	To identify the fatty acids	Agilent Technologies, Milan, Italy
28	Rheometer	To analyse the rheology	Anton Paar Modular Compact Rheometer (MCR) 52, Austria
29	Digital refractometer	To analyse the TSS	HI 96801 Digital Refractometer
30	Colour measuring Spectrophotometer	To measure the colour	Konica Minolta Spectrophotometer CM-5, Japan

4.2 Methods

The flow chart for the outline of the entire study methodology is represented in Fig. 10.

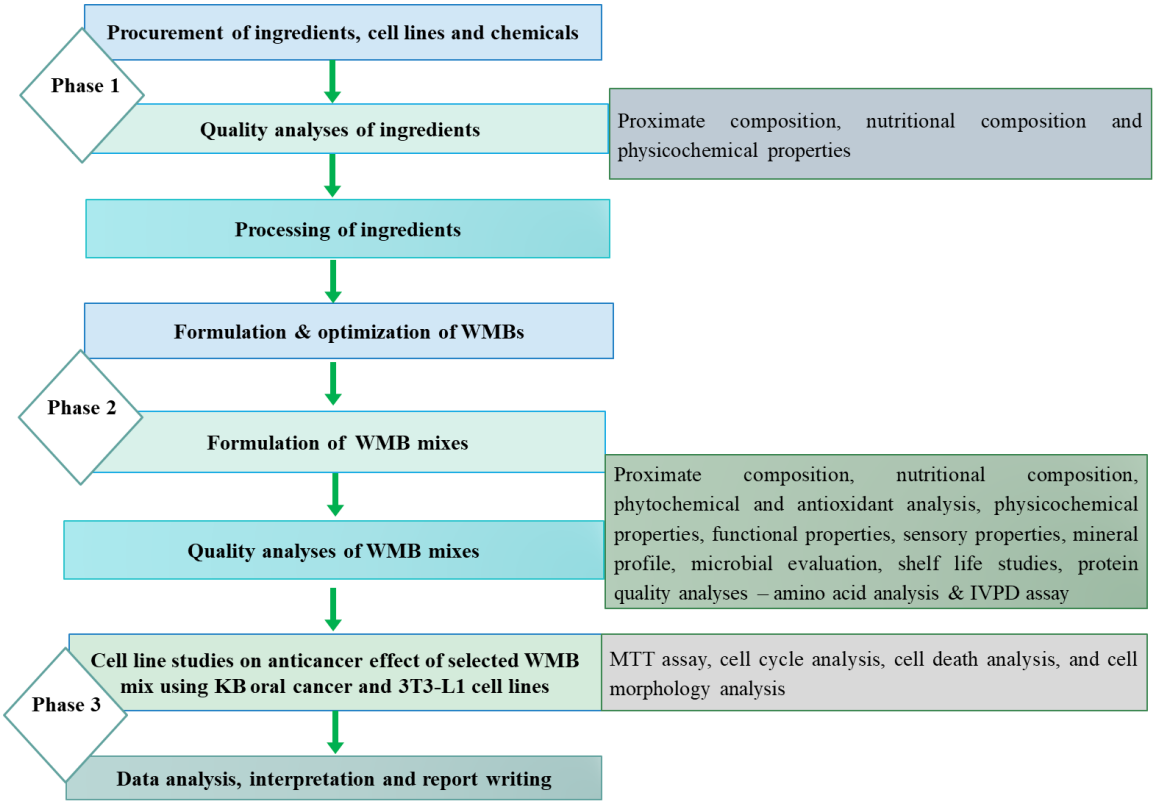


Figure 10. Flow chart for the outline of the study methodology

4.2.1 Study area

The study was carried out in the Department of Clinical Nutrition and Dietetics, Sri Devaraj Urs Academy of Higher Education and Research, (SDUAHER), Kolar, Karnataka, as well as Department of Fruit and Vegetable Technology and Department of Biochemistry, Central Food Technological Research Institute (CFTRI), Mysore, Karnataka.

4.2.2 Procurement of ingredients

1. Whey

Fresh cheese whey was obtained during the cheddar cheese preparation from KOMUL, Kolar.

2. Tomato

Arka Apeksha variety tomatoes were procured from a farmer from Maddur, Mysore, Karnataka, who had collected Arka Apeksha tomato seeds from Indian Institute of Horticultural Research (IIHR), Bangalore and harvested it (Fig. 11).

3. Banana

Well ripened nendran bananas without blemishes and injuries were collected from a farmer, Kannur, Kerala.

4. Flaxseed

Flaxseeds (brown coloured variety) were collected from the University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra (GKVK) store, Bangalore.

5. Sesame seed

Sesame seeds (black coloured variety) were procured from Indian Institute of Oilseeds Research (IIOR), Hyderabad.

Figure 11. Procurement of Arka Apeksha tomatoes from farm land

4.2.3 Procurement of cell lines and chemicals

1) KB OC cell lines and 3T3-L1 cell lines were procured from National Centre for Cell Sciences, Pune.

2) All chemicals, reagents, chemical kits, and standards used in the experiments were of analytical grade and purchased from Sigma Aldrich, and Hi-Media chemical suppliers, Bangalore, Karnataka.

4.2.4 Quality analysis of ingredients

The nutritional and physico-chemical analysis of whey, tomato, banana, flaxseed and sesame seed oil cakes were carried out initially to make sure the quality of ingredients selected and to ensure the final product meets desired overall properties and standards. The ingredients were

evaluated for proximate composition parameters such as moisture, fat, protein, ash, and carbohydrate as well as other nutritional components like TPC, Vit C, lycopene, and βC (for those ingredients that are known to be abundant in these components) and physico-chemical parameters like TSS, acidity and pH. The detailed procedures are given below in section 4.2.8 under quality analyses.

4.2.5 Processing of ingredients

1) The collected cheese whey samples were filtered first using a clean muslin cloth and then pasteurized at 72ºC for 15 seconds and cooled to 37 ºC (13). After cooling, it was kept at refrigerator temperature (4±1°C) until further use (Fig. 12).
2) Tomatoes were washed initially thoroughly with potable water and crushed using a fruit mill. The resulting crushed pulp was processed under steam jacketted kettles for heating, followed by the extraction of fine pulp (without seeds and skin) from crushed fruits using a fruit pulper, filtered using a muslin cloth and kept in refrigerator until further use (Fig. 13).
3) Well ripened nendran bananas were washed and cleaned thoroughly with potable water including the peel to remove dust, dirt and other foreign materials and kept for boiling in an electric hot plate for 10 minutes followed by the removal of peel and mashing of the pulp and kept in refrigerator until further use (Fig. 14).
4) The flaxseeds and sesame seeds were directed for extraction of oil using a mechanical cold press oil extraction machine separately and collected the chunks of oil cakes obtained as by-product. The resulting FOCs and SOCs obtained were cleaned from foreign material, ground and sieved to obtain fine powder and preserved until further processing in refrigerator until further use (Fig. 15).
5) All the processed ingredients were packed airtight in LDPE films and sealed and kept in refrigerator conditions until further use.

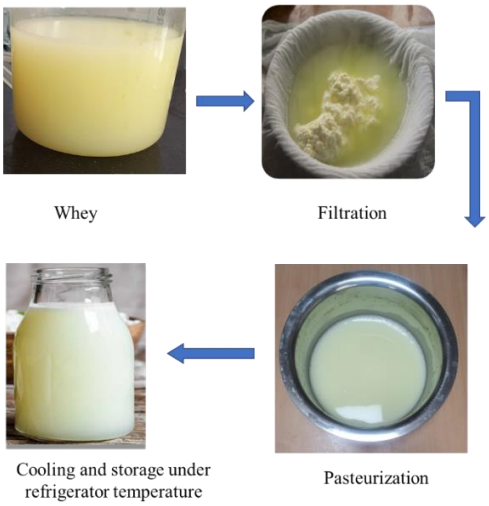


Figure 12. Illustration of whey processing

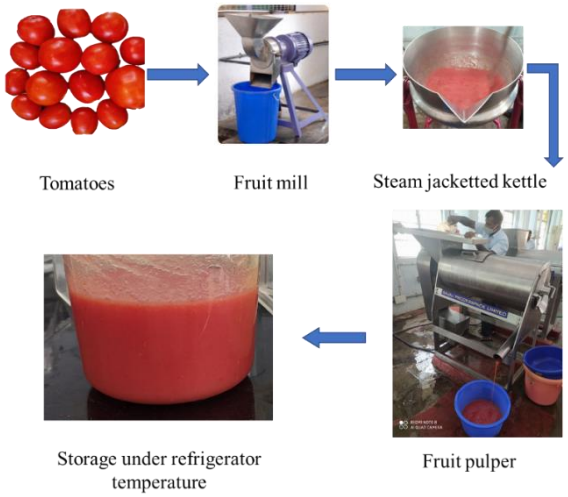


Figure 13. Illustration of tomato pulp processing

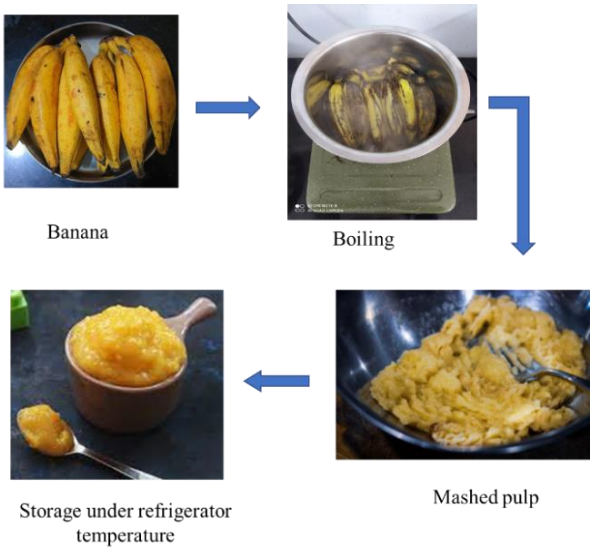


Figure 14. Illustration of banana pulp processing

Figure 15. Illustration of FOC and SOC processing

4.2.6 Formulation and optimization of WMB

The different whey-based multi-ingredient beverages (WMBs) were formulated after several iterations of sensory acceptability tests using ingredients of whey, tomato, banana, flaxseed, and sesame seed oil cakes and these were blended and pasteurised to form five test formulations (C, T1, T2, T3, and T4) with varying ratios on a laboratory scale (Table 7). The different beverage blends were amended based on sensory and physico-chemical evaluation to optimize the final product. The optimization of WMB involved several initial experiments with different ratios of ingredients to select the final formulation and also with respect to the results of various quality analysis of developed WMBs and sensory evaluation. The effect of different levels of whey and tomato pulp, on the sensory characteristics (flavour, body and texture, colour and appearance and overall acceptability), and other quality analysis including nutritional attributes determined the final optimization of the WMB mix. The final optimized formulation among the four test formulations was subjected for further studies of mineral profile, fatty acid and

amino acid analysis, microbial evaluation, shelf-life studies, protein digestibility as well as cell line analyses against the control sample.

Table 7. Formulation of WMB

Treatment	Whey (%)	Tomato (%)	Banana (%)	FOC (%)	SOC (%)
Control (C)	0	88	10	1	1
T1	45	43	10	1	1
T2	65	23	10	1	1
T3	75	13	10	1	1
T4	85	3	10	1	1

C-control; T1- Test formulation 1; T2- Test formulation 2; T3-Test formulation 3; T4- Test formulation 4.

4.2.7 Formulation of WMB mixes

All the developed WMB formulations were undergone homogenisation thoroughly before being drum dried at a temperature of 100°C and a drum speed of 20 revolutions per minute (rpm) to prepare WMB flakes (52). The drum-dried flakes were then made into fine powder using a mixer grinder (Make: Panasonic MX-AC555 1000-Watt mixer grinder) for 3 ± 0.2 min at high speed to make WMB mix and stored in polythene airtight bags at -20°C, protected from light until further use, and estimated for various quality analyses, and cell line studies. The pictorial illustration of formulation of WMB and mixes are given in Fig. 16 below.

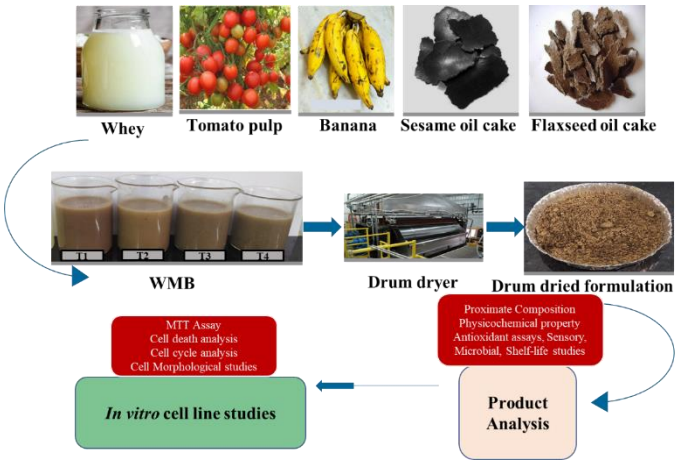


Figure 16. Illustration of formulation of WMB and mixes

4.2.8 Quality analyses

The quality analyses of ingredients as well as WMB mixes were carried out involving proximate composition analyses, nutritional composition analyses, and physico-chemical property analyses, with microbial evaluation, shelf-life studies, amino acid analyses, fatty acid analysis, protein digestibility studies and cell line analyses done only for the final optimized formulation. The ingredients were evaluated for proximate composition parameters such as moisture, fat, protein, ash, and carbohydrate as well as other nutritional components like TPC, Vit C, lycopene, and βC (for those ingredients that are researched to be rich in these other nutritional components) and physico-chemical parameters like TSS, acidity and Ph, while the developed WMB mixes were evaluated for above mentioned analyses as well as other parameters including water activity (water activity meter), total solids (moisture analyser), viscosity (viscometer), colour (spectrophotometer), rheology (rheometer), and sensory properties. Besides, the final optimized formulation (T1 WMB mix) among the four test formulations was subjected for further studies of mineral profile, amino acid analysis, microbial evaluation, shelf-life studies, protein digestibility as well as cell line analyses against the control sample.

4.2.8.1. Proximate composition analysis

1. Moisture

The moisture content was determined by drying the samples in a moisture analyser (Denver Instrument IR 35, Germany) (Fig. 17) at 120°C which is based on the loss on drying principle, and provide reliable results in minutes instead of hours. Percentage moisture and percentage solid values were obtained using this method. About 2 gm of samples were kept inside the analyser and moisture content was recorded as displayed as percentage (%).

2. Ash

The ash content was determined according to the standard methods by the “Association of Official Analytical Chemists” (AOAC), 2005 using a muffle furnace (129). At first, a clean

and empty crucible was ignited in muffle furnace for 6-8 h at 550°C temperature to confirm all the contaminants are burnt off. It was then taken out, cooled in a desiccator and noted the weight (W1). About 3-5 gm of samples were then weighed into the crucible, ignited in muffle furnace for 6-8 h at 550°C temperature. The completely ignited and ashed sample was taken out of the muffle furnace, cooled and weighed again (W2) (Fig. 18). The emergence of gray-white ash signifies the total oxidation of all organic materials within the specimen. The total ash content was calculated using following formula and expressed as percentage (%).

$$\% \text{ Ash} = \frac{\text{Difference in weight of ash} \times 100}{\text{weight of sample}}$$

Where, difference in weight of ash = W2-W1

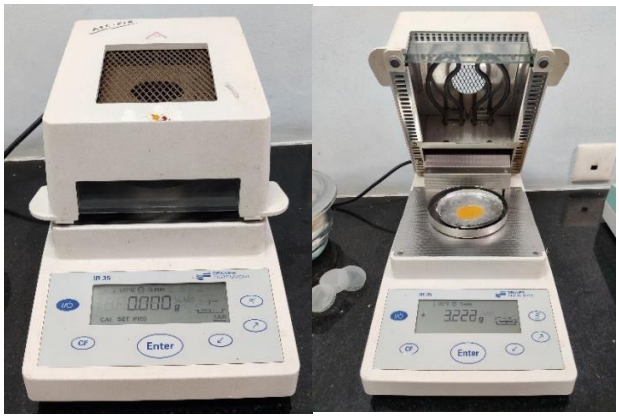


Figure 17. Moisture analyser (Denver Instrument IR 35, Germany)

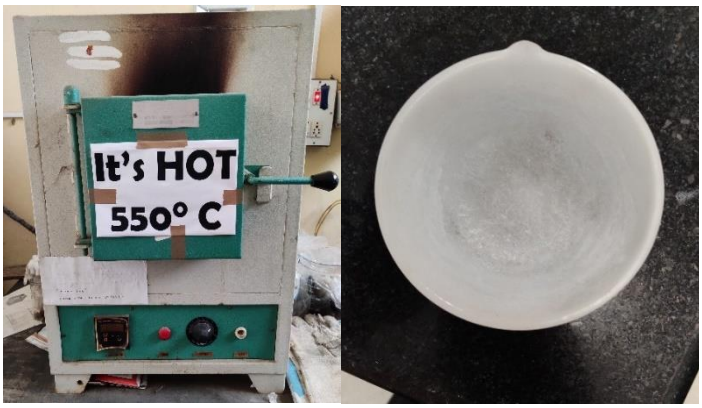


Figure 18. Muffle furnace and crucible

3. Fat

Petroleum ether was used as the extraction solvent to assess the fat content using the Soxhlet method (997.09) of the AOAC 2005, wherein the samples were subjected to heating in the round bottom flask containing boiling chips on a water bath set at 60°C for 6 –8 hours, and the total oil yield was calculated gravimetrically by the following formula (129).

$$\% \text{ Fat } = \frac{\text{Weight of ether extract} \times 100}{\text{weight of sample}}$$

4. Protein

Thermo Scientific Flash 2000 Organic elemental analyser, Italy, was used for nitrogen estimation which works on the Duma's combustion principle and presented the results as percentage (%) protein (Fig. 19). The estimated nitrogen content was multiplied by 6.25, the normal nitrogen-to-protein conversion factor, to get the protein content.

Fig 19. Flash 2000 Organic elemental analyser, Italy

5. Carbohydrate

The difference method (100 - the sum of moisture, ash, fat, and protein) was used to calculate the total carbohydrate content (84).

6. Energy value

The Atwater general factor (fat x 9 + carbohydrate x 4 + protein x 4 kcal/100 g) (FAO, 2003) was used to determine energy content of the samples (130).

4.2.8.2. Nutritional composition analysis

4.2.8.2.1 Vit C

Vit C was extracted from the samples using an extraction solution made of 3% metaphosphoric acid, 8% acetic acid, 1 mM EDTA, and 0.3N H_2SO_4. The supernatant of samples was collected and filtered through 0.45µm nylon filters and used for the HPLC analysis (Shimadzu, 10AV UV–VIS detector) equipped with a C18 chromatographic column (250mm × 4.6mm). About 1mM each of NaH_2PO_4 and EDTA at pH 3.00 was used for the mobile phase in isocratic mode. Twenty microliters of the sample were injected, and the flow rate was set at 1 mL min^{-1}. The quantification was carried out using ascorbic acid as an external standard, and a calibration curve was prepared (131).

4.2.8.2.2 Lycopene and β-carotene

The lycopene and βC was estimated spectrophotometrically (Microplate reader, Thermo Scientific evolution 220, CA, USA) following the method of Nagata and Yamashita 1992, using the extraction solution with acetone-hexane (4:6) at once, and then the optical density of the supernatant was measured at 505 and 453 nm respectively (132).

4.2.8.2.3 Extraction of bioactive compounds

About 1 g of WMB mix was dissolved in 25 ml of 80% methanol and kept for extraction in an incubator cum shaker at 250 rpm at room temperature for 6 –8 hours. After the incubation, it was filtered through Whatman filter paper no. 5 and stored at 4°C until further analysis. Flow chart for the preparation of sample extracts for antioxidant assays is given below in Fig. 20.

4.2.8.2.4 Total phenolic content

The Folin-Ciocalteu method (133) was slightly modified and used to estimate the total phenols in the extracts. Briefly, aliquots of 0.1 ml were combined with distilled water and made up to 0.5 ml. Further, about 2.5 ml of Folin-Ciocalteu's reagent was added and agitated. After 4 minutes, about 2.0 ml of 7.5% (w/v) sodium carbonate was added and kept for incubation at

room temperature for 2 hours, and an intense blue colour was noticed. After the incubation, absorbance was measured at 760 nm using a spectrophotometer, which is used for lycopene analysis. The reagent blank with solvent was used as a blank, and gallic acid was employed as a reference standard. The results of TPC for WMB mix were calculated using a standard calibration curve of gallic acid by the following formula and represented as mg of gallic acid equivalent (GAE) weight per 100 grams of dry extract.

$$\text{Total Phenolic Content (TPC) (mg/100g)} = \frac{C \times OD \times V}{M}$$

Where, C-Concentration of gallic acid established from the standard curve (mg/mL)

V-Volume of extract solution (mL)

OD-Absorbance

M-Weight of the extract (g)

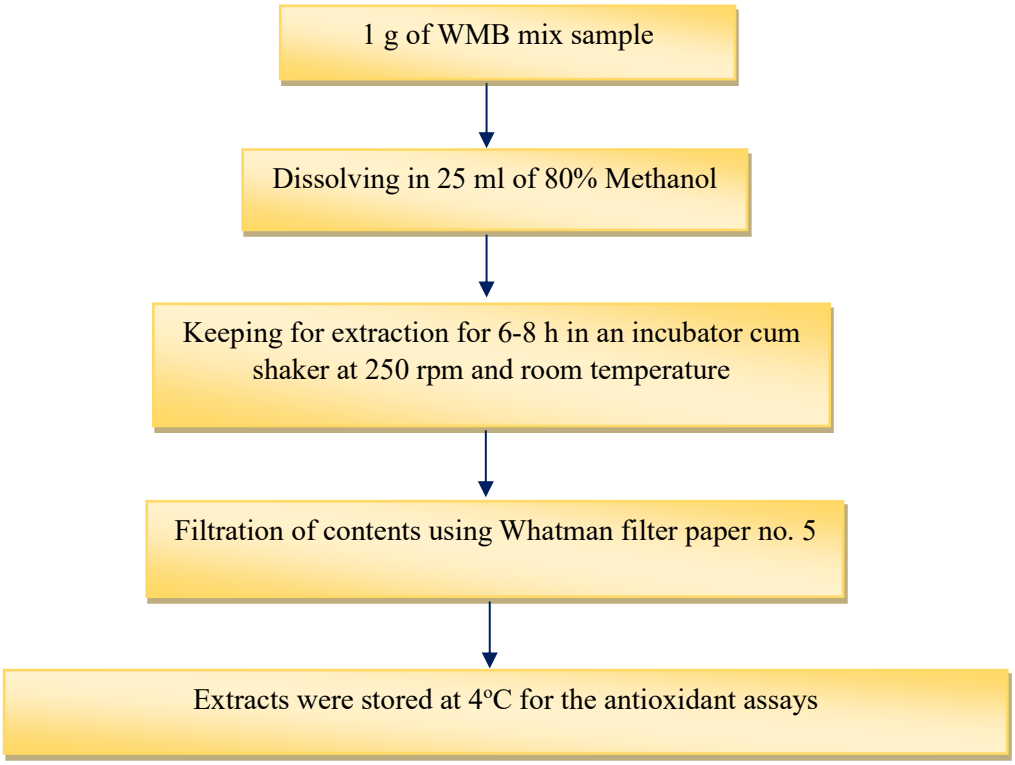


Figure 20. Flow chart for the preparation of sample extracts for antioxidant assays

4.2.8.2.5 Total antioxidant capacity

The total antioxidants of WMB were analysed by Trolox equivalent antioxidant capacity assay reported by Van Den Berg et al. 1999 (134). A 3 ml mixture of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate was added in equal proportion to 0.3 ml of WIBM extracts. The reaction tubes were thoroughly mixed and then incubated for 90 min at 95 °C. Thermo Scientific Evolution 220, CA, USA, microplate reader was used to measure the absorbance at 695 nm after the tubes had been further cooled. 1 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as reference standard at different concentrations (100, 200, 300, 400, and 500 µM). The TAC of WMB were expressed as mM of Trolox equivalents (TE) per gram of dry extract.

4.2.8.3. Physicochemical property analysis

Physicochemical characteristics evaluation was done for various parameters including water activity (water activity meter), total solids (moisture analyser), total soluble solids (refractometer), pH (pH-metre), titratable acidity (titrimetric method), viscosity (viscometer), colour (spectrophotometer), and rheology (rheometer).

4.2.8.3.1 Water activity (a_w)

Novasina Lab Touch-aw, Switzerland digital water activity meter (Fig. 22) was used to measure the water activity of the samples. The humidity above the sample is measured in % relative humidity, immediately after reaching the humidity equilibrium and converted electronically into water activity (aw) value.

Figure 21 & 22. Water activity meter closed and opened

4.2.8.3.2 Total soluble solids

The total soluble solids content (TSS) was determined using a digital refractometer (HI 96801 refractometer) (Fig. 23) and the results were expressed as °Brix. A drop of the homogenized sample was placed on the refractometer and displayed TSS content was recorded.

Figure 23. HI 96801 Digital Refractometer

4.2.8.3.3 pH measurement

The pH values of the samples were determined by a digital pH-metre with a suitable electrode (Agilent Technologies 3200P pH meter) (Fig. 24). Prior to measurement, the pH meter was calibrated using the commercial buffer solutions, pH 10, pH 7 and pH 4 by dipping the probe with buffers in accordance with the manufacturer's recommendations. The glass electrode was dipped in the sample solution to record the potential difference between the glass electrode and the reference electrodes. The potential difference is used to measure the hydrogen ion concentration indicating the pH. The electrode probe was washed with distilled water and wiped with dry tissue paper. Following stabilization, the pH of a sample containing around 30 mL taken in a beaker was measured using a pH-meter. After calibration the electrode was immersed in the sample until a stabilized value was achieved which was noted down as the pH of the sample, subsequently it was washed, wiped dry and used for subsequent pH readings.

Figure 24. Digital pH meter

4.2.8.3.4 Titratable Acidity

Titration with a basic reagent, NaOH, (0.1 N) was employed to assess titratable acidity (TA) with phenolphthalein as indicator. The results were expressed as percentage (%) of lactic acid or citric acid based on the sample. TA was measured by titrating about 2 g of sample in 20 ml of distilled water against 0.1 N NaOH using few drops of 1% phenolphthalein indicator. The endpoint was a permanent pink colour appearance and the titre value was noted. The percentage of TA was calculated using the following formula

$$\% \text{ Titratable Acidity} = \frac{\text{Titre value} \times \text{Normality} \times \text{Volume of alkali made up to} \times \text{Equivalent weight of acid}}{\text{Volume of sample} \times \text{weight or total volume of sample taken for estimation}} \times 100$$

4.2.8.3.5 Total solids

Total solids content (%) was measured using the same moisture analyser used for moisture estimation mentioned earlier.

4.2.8.3.6 Viscosity

A calibrated digital viscosity meter (Brookfield DV-II+Pro, USA) (Fig. 25) was used to determine the viscosity of samples. The operations were done in at 100 rpm with spindle

number 4 (RV4) at room temperature for 120 seconds and the average readings were noted in centipoise (cP).

Figure. 25 Viscometer

4.2.8.3.7 Colour

The colour (L*, a*, b* value) was measured using colour measuring system, Konica Minolta Spectrophotometer CM-5, Japan, (Fig. 26) using L*, a*, b* scale. The results were expressed as lightness L*, a* and b* values which are the chromaticity coordinates (a* from red to green; b* from blue to yellow). L* denotes darkness to lightness on a 0-100 scale from black (0) to white (100). The other two coordinate a* and b* represents redness (+a) to greenness (-a) and blueness (+b) to yellowness (-b), respectively. Three readings of the samples were taken and the computerized data were obtained from the system.

Figure 26. Colour measuring system (Spectrophotometer CM-5)

4.2.8.4. Functional properties

4.2.8.4.1 Solubility

Solubility was determined in which 5 g of sample was suspended in 50 mL of distilled water at 30°C. The suspension was occasionally stirred for 30 min and centrifuged at 9,500 rpm for 10 min. The supernatant was drained into a pre-weighed evaporating dish and dried at 105°C to constant weight. The weight of the solids recovered after drying was used to calculate the water solubility (135).

4.2.8.4.2 Wettability

Wettability was determined by considering the time (s) required for the powder to become wet and penetrate the surface of the distilled water at room temperature (135).

4.2.8.4.3 Dispersibility

Dispersibility was determined following a modified method in which beverage mix (1 g) was added into 10 mL distilled water in a beaker and stirred vigorously with a spoon for 15 s. The rice beverage solution was filtered and then transferred to a pre-weighed Petri plate, dried for 2 hr in a hot air oven at 130±1°C (135). The dispersibility was calculated as:

$$\% \text{ Dispersibility} = [10+a\times\%TS/a\times100b/100]$$

where, a is the amount of powder, b is the moisture content of the powder, and TS is the dry matter in the rice beverage

4.2.8.4.4 WAI and WSI

Water absorption index (WAI) and water solubility index (WSI) was determined in which 1 g of sample was weighed and placed in a centrifuge tube. Then 6 ml of distilled water was added for suspension. The tubes along with the samples were heated in shaking water bath at the temperature of 80°C for 30 minutes. The solution was centrifuged at 2500rpm for 10 minutes. After the centrifugation, the supernatants were carefully poured into Petri dish for drying at 105°C for 10 hours in an oven, while the sediments were weighed as such (136).

The WSI of samples was carried out in which 1 g each of the samples and 10 ml of water were mixed in a 15 ml plastic centrifuge tube and were immersed in water bath for 30 min at 37°C. This was then centrifuged at 4000 rpm for 10 min after which the supernatant was collected in a pre -weighed beaker and the residue was weighed after the water was evaporated at 105°C; the percentage of residue with respect to the amount of sample used was taken as the WSI (136).

$$\% \text{ WAI} = \frac{\text{Weight of wet sediment}}{\text{Dry weight of sample}}$$

$$\% \text{ WSI} = \frac{\text{Weight of dry supernatant}}{\text{Dry weight of sample}}$$

4.2.8.4.2 Rheological properties

The flow behaviour and viscosity of beverages and the effect of temperature on consistency coefficient (k) and flow behaviour index (n) of different test formulations were determined using a Rheometer (Anton Paar Modular Compact Rheometer (MCR) 52, Austria). The formulations were analysed at two suitable consumption temperatures of 10°C and 25°C.

4.2.8.5 Sensory evaluation

The sensory analysis of WMB was conducted by 20 semi-trained panellists who were recruited depending on various selection criteria, including their interest and availability as well as their allergic history, familiarity with the product and by adopting 9-point hedonic scale scoring on an increasing scale from 1 (extremely disliked) to 9 (extremely liked) (Table 8) on 0^{th} day, 45^{th} day and 90^{th} day (after three months storage period) (23). Prior to be enlisted in the sensory evaluation test, all the recruited panellists were briefed about the nature of the product, study and methodology to enable them to make an informed decision. An aliquot of 10 mL of WMB was served in transparent bottles and the panellists were asked to record their observations on different study parameters such as appearance/colour, flavour, consistency/texture, taste,

mouthfeel/after taste and overall acceptability on the sensory score card provided (Appendix 2).

Table 8. 9-Point Hedonic scoring system

Panellist hedonic rating	Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

4.2.8.6 Mineral profile of T1 WMB mix

Using atomic absorption spectroscopy (AAS)/inductively coupled plasma atomic emission spectroscopy (ICP-AES), the mineral compositions of C and T1 WMB mixes were ascertained for both macro and micro minerals, including potassium, sodium, calcium, magnesium, and zinc, in accordance with AOAC 2005 methodology (129) (Table 9). AAS is predicated on free atoms in their gaseous state absorbing UV-visible light. The mineral particles are vaporized and atomized by heating the ash solution inside the apparatus. After passing a radiation beam through the atomized sample, the amount of radiation absorbed at particular wavelengths that correlate to the mineral composition is determined.

4.2.8.6.1 Preparation of ash solution for estimation of minerals

About 5 ml of concentrated nitric acid (HNO_3) was added to the obtained greyish-white ash samples from ash content determination and evaporated, digested, cooled, and filtered through Whatman No. 42 filter paper and made up to 50 ml with 2% HNO_3 (Fig. 27).

Figure. 27 Preparation of ash solutions

Table 9. Wavelengths used for mineral estimation

SL.NO.	Minerals	Wavelength (nm)
1.	Calcium (Ca)	422.7
2.	Zinc (Zn)	213.9
3.	Magnesium (Mg)	285.2
4.	Potassium (K)	766.5
5.	Sodium (Na)	589.6

4.2.8.6.2 Determination of mineral profiles

A portion of the greyish-white ash solution was introduced into the AAS/ICP-AES instrument for the analysis of minerals including Ca, Mg, K, Na, Cu, Mn and Zn. The calibration of the ICP-AES was carried out using a working standard created from a commercially available multielement standard solution (100 mg/L, Merck, Germany). Optimal parameters for the ICP-AES instrument, such as the most suitable wavelength, argon gas flow, plasma stabilization, and others, were chosen for the analysis of the minerals. Measurements were conducted within the linear range of the working standards employed during calibration. The working conditions for the ICP-AES were as follows: instrument - ICP-AES (ACTIVA-M, Horiba Jobin-yvon, USA), power - 1000 W to 1200 W, plasma gas flow - 12 L/min to 16 L/min, auxiliary gas flow

- 0.8 L/min, plasma burning height - 5 mm to 22 mm, reading time - 1 sec to 10 sec (maximum of 60 s), and flow time - 2-3 sec (maximum of 10 s). By comparing the emission of a mineral with known amounts of the mineral displayed on a calibration curve, the concentration of mineral in a sample is quantified. The obtained mineral concentrations were denoted in mg/100 g of WMB mix.

4.2.8.7 Microbiological evaluation

The microbiological evaluation with regard to total bacterial count, total coliform count, total yeast and mold count and total salmonella count was enumerated in the T1 WMB mix sample under the laminar flow (Fig. 28) at 0^{th} day and after 90 days of storage using nutrient agar (NA) media for total bacterial count, Eosin methylene blue agar (EMB) for total coliform count; Rose Bengal agar (RBA) for total yeast and mold count, SS media for total Salmonella count. The sample was accurately weighed, homogenized and serially diluted in sterile physiological saline (0.85% NaCl w/v) and appropriate dilution was plated on selective media. Plates were incubated at 37°C for 24-48 h until visible colonies were formed. For yeast and mold count, plates were incubated at 30°C for 4-5 days. The colonies were counted and the results were expressed as colony forming unit/g of the sample (CFU/g). Experiments were conducted in triplicates and mean values were calculated.

Sterilized experimental glass wares and plastic wares were used. 70% ethanol treatment of floor and Laminar Air Flow (LAF) chamber was done to avoid contamination. General safety precautions such as wearing lab coat, gloves, head cap, face mask and lab footwear were used to ensure adequate personal health and safety. Culture plates and sample were discarded by decanting at 121°C for 20 min at 15 lb pressure.

Figure 28. Laminar air flow chamber

4.2.8.8 Shelf-life studies of WMB mix

T1 WMB mix was stored in a metallized pouch (heat sealed) for 90 days' time period at refrigerated condition (4±1°C) and analysed for various physicochemical and sensory parameter analysis on every 45 days interval. Samples were tested regularly on every 45 days for changes in chemical composition (e.g., TSS, acidity, pH, and water activity) and sensory scores to assesses changes in taste, aroma, and appearance.

4.2.8.9 Protein quality analyses of T1 WMB mix

The protein quality of the developed T1 WMB mix was determined by estimating the amino acid content, and *in vitro* protein digestibility (IVPD)methods.

4.2.8.9.1 Amino acid analysis of T1 WMB mix

The amino acids (EAAs such as histidine, threonine, methionine, valine, phenylalanine, isoleucine, leucine and lysine as well as non-EAAs such as aspartic acid, glutamic acid, serine, glycine, arginine, alanine, tyrosine, cystine, and proline) content was analysed by ultra high-performance liquid chromatography (UHPLC), employing method described by Horanni and Engelhardt 2013 with slight modifications (138–140). The essential amino acid tryptophan was

broken down during acid hydrolysis, making it unable to detect and thus it was not measured and the non-essential amino acids asparagine and glutamine, were transformed into aspartic acid and glutamic acid, respectively (141).

4.2.8.9.1.2 Sample preparation

Initially, a 50 mL schott glass bottle was filled with 0.5 g of the T1 WMB mix sample. After adding 20 mL of 6 N hydrochloric acid (HCl) solution, the mixture was hydrolysed for 24 hours at 120 °C in an oven. After hydrolysis, the acid was removed by rotary evaporation and sample was resuspended in 25 mL of sodium citrate buffer at pH 2.2.

4.2.8.9.1.3 UHPLC condition

The amino acids were identified using a pre-column derivation with ortho-phthalaldehyde (OPA) (Sigma®, St. Louis, MO, USA). The UHPLC instrument (Nexera UHPLC (Shimadzu Corporation, Japan)) was equipped with a SIL-30AC autosampler, a 5.0 µm (250 mmL. × 4.6 mm I.D.,) reverse-phase C18 column (Shim-pack GIST), and a fluorescence detector (RF-20AXL, Shimadzu Corporation, Japan) monitoring excitation and emission wavelengths at 350 nm and 450 nm, respectively. The mobile phase consisted of buffer solution (A) and (B). Mobile Phase A was 25 mmol/L phosphate potassium buffer adjusted to pH 6.9 and B was acetonitrile: methanol: water, 45:40:15, (v/v). The total running time per sample was 50 min and the column temperature was kept at 40°C temperature. The mobile phase gradient time program used in the separation of amino acids is B Conc.10 % (0.01) → 11 % (0.35 min), → 45 % (35.00 min) → 100% (40.00-45.00 min), → 10 % (50.00 min), at 1.0 mL/min flow and the injection volume was 10 µL.

4.2.8.9.1.4 Preparation of derivatization reagents

1. Mercaptopropionic Acid: 3- Mercaptopropionic Acid 10 µL in 0.1 mol/L borate buffer (pH 9.2) 10 mL, 2. Ortho-phthalaldehyde (OPA) solution: OPA 10 mg in 0.1 mol/L borate buffer

(pH 9.2) 5 mL, 3. 9-fluorenylmethyloxycarbonyl (FMOC) – Acetonitrile solution: 9-fluorenylmethyloxycarbonyl 4 mg in Acetonitrile 20 mL.

4.2.8.9.1.5 Derivatization procedure

Mercaptopropionic Acid, OPA and sample was taken in (µL) ratio of 45:22:7.5 and thoroughly mixed and kept for 1 min. Then added 10 µL of FMOC to it and sample was injected to the UHPLC using autosampler.

4.2.8.9.2 *In vitro* Protein Digestibility assay

With minor adjustments, the IVPD test was carried out for T1 WMB mix test formulation and control sample using the methodology outlined by Almeida et al. 2015 (142). In short, 15 mL of 0.1 mol equi/L HCl containing 1.5 mg/mL pepsin was used to suspend 250 mg of each sample or 250 mL of milli-Q water (for the blank) and the mixture was then incubated for three hours at 37°C in a water bath. Following neutralization with the addition of 7.5 mL of 0.5 mol equi/L of NaOH, the pepsin hydrolysis stopped. After that, 10 mL of 0.2 mol/L phosphate buffer (pH 8.0) containing 10 mg of pancreatin and 1 mL of 0.005 mol/L sodium azide were added to start the pancreatic digestion. The mixture was then incubated at 37°C for the entire night. Following the pancreatic hydrolysis, 1 mL of 10 g/100 mL trichloroacetic acid was added, and the mixture was centrifuged for 20 minutes at 10,000 rpm. After collecting the supernatant, the Kjeldahl AOAC 2005 (129) technique was used to estimate the total protein concentration based on the nitrogen content. The reference used was casein. The following formula was used to calculate the IVPD values:

$$\% \text{ Protein Digestibility } = \frac{SN-BN \times 100}{SN}$$

where SN and BN represent the nitrogen content in the sample and in the blank, respectively.

4.2.9 Anticancer activity assays of T1 WMB mix

4.2.9.1 Cell culture and treatment

The cell line assays were performed by making use of the KB OC cells and the 3T3-L1 cell line, which is a murine fibroblast cell, as non-tumoral lineages 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 IU/ml), and streptomycin (100 μg/ml) with pH 7.4, in a humidified atmosphere of 5% CO2, and at 37°C temperature until confluent. Cell lines in the same media devoid of extract served as controls.

4.2.9.2 Determination of cytotoxicity by MTT assay

The cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, USA) dye reduction method (143). Firstly, about 80% of confluent cells from both cell lines were trypsinised, and then each well was loaded with medium at a density of 5×10^4 cells in 100 μL and incubated at 37°C for 24 h (5% CO_2). The media was withdrawn using a pipette from this cell suspension and restored with 100 μL of DMEM containing different concentrations of treated samples, i.e., doxorubicin (100 μM – 1.56 μM), T1 (320 mg mL^{-1} –10 mg mL^{-1}) along with the test control sample and individual ingredient samples of WP, tomato, banana, and SOC as well as FOC 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 (0.5 mg mL^{-1} in 1X phosphate buffered saline (PBS) filtered through a 0.2 μM filter) 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 rapidly by adding 100 μL of dimethyl sulfoxide (DMSO). Doxorubicin-treated and untreated cells served as standard and control, respectively. The absorbance was measured at 590 nm using a microplate reader

(Thermo Scientific Evolution 220, CA, USA), IC_{50} values and percent cell viability was calculated using equation (4) (144).

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (4)$$

4.2.9.3 Cell cycle analysis

Cells were separately plated in 6-well microplates with approximately 3 mL of cell suspension (5×10^5 cells/well) and subjected to incubation for 24 h at 37°C and 5% CO_2. Following the exposure time, 1 mL of doxorubicin (standard) and T1 were added after the growth medium was removed by suction. Upon that, the growing medium was incubated in humified conditions. After trypsinization, the treated cells were centrifuged for 10 minutes at 2000 rpm and then continually washed with PBS. Post that, the cell pellet was fixed with 700 µL of ethanol and kept at − 20°C for an extra hour. The cells were twice chilled with PBS before being centrifuged for 10 minutes at 4000 rpm. Prior to being examined using a flow cytometer (Beckman Coulter, USA), the final cell pellet was also resuspended in 1 mL of PBS containing 50 mg mL^{-1} of propidium iodide, 50 mg mL^{-1} of RNase A, and 0.1% Triton X-100 and allowed to incubate for 30 minutes in the dark.

4.2.9.4 Cell death analysis

Furthermore, using the Annexin V-FITC apoptosis detection kit (Invitrogen, USA) in accordance with the manufacturer's instructions, the T1 treated KB OC cells and the previously described control sets were subjected to Annexin V-FITC staining analysis(144). Briefly put, PBS was used to wash the KB OC cells treated with T1 and the control groups before they were again suspended in binding buffer (1X, 0.2 mL). Annexin V-FITC was added in an amount of about 0.05 mL to 0.195 mL of cell suspension, following a thorough mixing process, and subjected to room temperature incubation for ten minutes. Following the designated time, the cell suspension was rinsed with 0.2 mL of the binding buffer and resuspended in 0.19 mL

of the same buffer. About 0.1 mL of propidium iodide was then added, mixed well, and allowed to incubate for 5 minutes in room temperature without light before being examined under a flow cytometer (Beckman Coulter, USA).

4.2.9.5 Cell morphology analysis

About two minutes of gentle mixing were spent for staining approximately 25 µL (1×10^5 cells) of treated and untreated KB OC cells each with acridine orange and ethidium bromide (AO-EtBr) (5 µL) in separate Eppendorf tubes. The stained cell suspension (10 µL) was fixed on a microscopic glass slide using a coverslip and viewed under a fluorescence microscope using a fluorescein filter (144).

4.2.10 Statistical analysis

The statistical analysis was performed via IBM SPSS version 26.0 software package for Windows (SPSS Inc., Chicago, IL, USA). All of the data is presented as the mean ± standard deviation (SD) of three replicates. Mean comparisons were performed by an analysis of variance (ANOVA) followed by a post hoc Duncan's Multiple Range Test (DMRT) to determine significance at $p \leq 0.05$. Significant relationship between different variables of bioactive components were tested by using Karl Pearson correlation coefficients with two-tailed test of significance and multiple regression analysis was employed to assess the individual impact of various bioactive components on the antioxidant profile.

CHAPTER 5
RESULTS

5.0 RESULTS

5.1. Quality analysis of ingredients

The ingredients obtained from various sources to develop the WMBs were analysed for required tests. Table 10 reveals distinct nutritional and physicochemical profiles for whey, tomato, banana, FOC, and SOC. The ingredients were evaluated for proximate composition parameters such as moisture, fat, protein, ash, and carbohydrate, as well as nutritional components like TPC, Vit C, lycopene, and βC and also physicochemical parameters like TSS, acidity, and pH. As per the results, whey had 93.60% moisture, 0.26% of fat, 0.77% protein, 0.50% ash, 4.87% of carbohydrates, 11.80 °Brix of TSS, 0.20% acidity, and 5.95 pH. Tomato samples recorded 94.25% moisture, 0.15% fat, 0.65% protein, 0.67% ash, 4.28% carbohydrates, 4.85 °Brix of TSS, 0.37% acidity, and 4.01 pH. Further, it also indicated the nutrient load of 0.58 mg GAE/g TPC, 16.50 mg/100g Vit C, 8.50 mg/100g lycopene, and 0.65 mg/100g of βC. Banana showed 63.08% moisture, 0.22% of fat, 0.69% protein, 0.78% ash, 35.23% of carbohydrates, 21.78 °Brix of TSS, 0.27% acidity, and 4.5 pH. Besides, it also had 0.38 mg GAE/g TPC, 15.30 mg/100g Vit C, and 1.16 mg/100g of βC. Also, FOC and SOC had 0.68 and 1.78% moisture, 34.08 and 14.62% of fat, 35.48 and 36.31% protein, 3.18 and 8.33% ash, and 26.58 and 38.96% of carbohydrates, respectively, which are measured on a dry matter basis, along with a very high TPC of 10.06 and 4.02 mg GAE/g. All values depicted in the table are the average of triplicates.

Among the ingredients, whey and tomato have high moisture content, while fat, ash, and protein content are substantially higher in FOC and SOC compared to others, as it was estimated on a dry weight basis. Carbohydrates are highest in banana, moderate in FOC and SOC, and lowest in whey and tomato. Further, FOC and SOC also show high levels of TPC, indicating antioxidant potential, along with tomato and banana. Tomato and banana are notable

for their Vit C content, with tomato also being a good source of lycopene and nendran banana of βC. Banana has the highest TSS, indicating more sweetness, compared to whey and tomato. These findings highlight the unique nutritional contributions of each ingredient, suggesting varied applications in food products based on their composition, particularly suitable for therapeutic beverage formulations.

Table 10. Nutritional and physicochemical property analysis of ingredients

Parameters (%)	**Whey**	**Tomato**	**Banana**	**FOC***	**SOC***
Moisture	93.60±0.06	94.25±2.52	63.08±0.34	0.68±0.15	1.78±0.01
Fat	0.26±0.05	0.15±0.07	0.22±0.11	34.08±0.22	14.62±0.32
Protein	0.77±0.25	0.65±0.18	0.69±0.13	35.48±0.38	36.31±0.26
Ash	0.53±0.05	0.67±0.06	0.78±0.16	3.18±0.11	8.33±0.18
Carbohydrate	4.86±0.19	4.28±0.39	35.23±0.09	26.58±0.17	38.96±0.28
TPC (mg GAE/g)	-	0.58±1.62	0.38±0.18	10.06±0.35	4.02±0.27
Vit C (mg/ 100g)	-	16.50±1.32	15.30±0.26	-	-
Lycopene (mg/100g)	-	8.50±0.86	-	-	-
βC (mg/100g)	-	0.65±0.03	1.16±0.48	-	-
TSS (°Brix)	11.80±0.10	4.85±0.18	21.78±0.75	-	-
Acidity	0.20±0.15	0.37±0.27	0.27±0.01	-	-
pH	5.95±0.06	4.01±0.01	4.5±0.01	-	-

All values are expressed in Mean±SD. '-' indicates value not determined, '*' indicates value on dry matter basis, FOC-Flaxseed oil cake, SOC – Sesame seed oil cake

5.1.2 Formulation of WMB formulations

The provided table 7 in the methodology section 4.2.6 outlines the composition of the formulation of WMB with varying proportions of whey, tomato, banana, FOC, and SOC. The control formulation (C) consisted of 88% tomato, 10% banana, and 1% each of FOC and SOC, with no whey included. In T1, 45% whey is introduced, reducing the tomato content to 43% while maintaining the banana, FOC, and SOC levels at 10%, 1%, and 1%, respectively. The T2 formulation further increases the whey content to 65%, with the tomato content lowered to 23% and the proportions of banana, FOC, and SOC remaining constant. T3 continues this trend by raising the whey content to 75% and reducing the tomato content to 13%, while keeping the other ingredients unchanged. Finally, T4 contained the highest whey content at 85% and the lowest tomato content at 3%, with banana, FOC, and SOC levels consistent across all formulations. This systematic variation in the whey and tomato content, while keeping the other ingredients constant, facilitates the evaluation of the effects of whey concentration on the nutritional, physicochemical, and sensory properties of the beverages.

5.1.3 Quality analysis of WMB mixes

The information regarding the quality analysis involving various nutritional and physico-chemical analyses of developed WMB mixes was carried out and is depicted in the following tables. All the four test formulations along with the control sample were evaluated for proximate composition parameters such as moisture, fat, protein, ash, carbohydrate, and energy, as well as nutritional components like lycopene, βC, TPC, and TAC, and physico-chemical parameters like TSS, acidity and pH, water activity (aw), viscosity, rheology, sensory analysis, functional properties, and microbial evaluation. Based on the results of these analyses, mineral profile, fatty acid analysis, amino acid analysis, protein digestibility assay, shelf-life studies, and cell line studies were carried out for the final optimized T1 WMB mix against the control sample.

5.1.3.1 Proximate composition of WMB mixes

Table 11 presents the proximate composition analysis of WMB mixes, including moisture, fat, protein, ash, carbohydrate, and energy content. Statistical significance among different formulations is indicated by different letters within each parameter. The moisture content of the test formulations varied from 3.90±0.08 to 4.49±0.10%, fat 0.17±0.02 to 1.58±0.32%, protein 3.79±0.08 to 4.79±0.02%, ash 3.14±0.11 to 4.71±0.08%, carbohydrate 85.70±0.39 to 87.80±0.17%, and energy 364.47±0.83 to 376.18±1.40 kcal, including the control formulation. The moisture content of the WMB mixes varied significantly across formulations. The control had a moisture level of 3.99±0.05%, which is slightly lower compared to T1 at 3.90±0.08%. However, this is significantly different from T2 (4.14±0.09%), T3 (4.32±0.04%), and T4 (4.49±0.10%), which exhibited progressively higher moisture contents. Fat content in the WMB mixes also showed significant variation. The control had the lowest fat content at 0.17±0.02%, which is significantly lower compared to T1 at 1.58±0.32% and other formulations, T2 (0.95±0.30%), T3 (0.64±0.01%), and T4 (0.78±0.12%).

Protein content also varied among the formulations, with the control showing the lowest value at 3.84±0.09%. T1 had the highest protein content at 4.79±0.02%, which is significantly different from T2 (4.54±0.11%), T3 (4.20±0.03%), and T4 (3.79±0.08%). The ash content decreased significantly from the control (4.71±0.08%) to T4 (3.14±0.11%). T1 had a higher ash content (4.03±0.05%) than T2 (3.69±0.06%), T3 (3.44±0.06%), and T4 (3.14±0.11%). Carbohydrate content increased significantly with higher whey percentages. T1 had the lowest carbohydrate content at 85.70±0.39%, while T4 had the highest at 87.80±0.17% among the test formulations. T2 and T3 also showed progressively higher carbohydrate levels compared to the control.

Energy content is highest in T1 at 376.18±1.40 kcal, which is significantly different from the lowest value for control (364.47±0.8 kcal) and other formulations. In summary, all parameters

showed highly significant differences across the formulations, with varying levels of moisture, fat, protein, ash, carbohydrates, and energy. These variations highlight the impact of formulation changes on the proximate composition of the WMB mixes.

Table 11. Proximate composition analysis of WMB mixes

Parameters %	C	T1	T2	T3	T4	P value
Moisture	3.99±0.05^{a}	3.90±0.08^{a}	4.14±0.09^{b}	4.32±0.04^{c}	4.49±0.10^{d}	**
Fat	0.17±0.02^{a}	1.58±0.32^{c}	0.95±0.30^{b}	0.64±0.01^{b}	0.78±0.12^{b}	**
Protein	3.84±0.09^{a}	4.79±0.02^{d}	4.54±0.11^{c}	4.20±0.03^{b}	3.79±0.08^{a}	**
Ash	4.71±0.08^{e}	4.03±0.05^{d}	3.69±0.06^{c}	3.44±0.06^{b}	3.14±0.11^{a}	**
CHO	86.88±0.25^{b}	85.70±0.39^{a}	86.68±0.39^{b}	87.41±0.09^{c}	87.80±0.17^{c}	**
Energy (kcal)	364.47±0.83^{a}	376.18±1.40^{c}	373.39±1.64^{b}	372.18±0.39^{b}	373.40±1.30^{b}	**

All values are expressed in Mean±SD. C- control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4; CHO - Carbohydrate. Values with same letters within a row are not significantly different by DMRT ($p < 0.05$), '**' indicates P value <0.001 which is highly significant at 1% level.

5.1.3.2 Nutritional Composition analysis

5.1.3.2.1 Bioactive component profile

The nutritional composition analysis was performed on the WMB mixes prepared, looking at the following bioactive components: Vit C, lycopene, βC, and TPC as well as antioxidant activity. The following tables and figures below show the outcomes that were determined by these criteria. The results showed significant variation among the control and the test formulations (T1, T2, T3, T4), which reflect the impact of different ingredient compositions on these bioactive compounds. It can be seen from the Table 12 and Figures 29 and 30 that

among the test formulations, T1 scored highest in all the bioactive component profiles when compared to other treatments. The control formulation exhibited the highest Vit C content (11.35±0.11 mg/100g), which significantly decreased across the test formulations from T1 to T4. This decline suggests that the reduction in tomato concentration (a key source of Vit C apart from banana) across these formulations is the main contributing factor when keeping the banana content constant. The significant difference among the formulations, as indicated by the P value, underlines the critical role of tomato in providing Vit C to the WMB mixes.

Lycopene content followed a similar trend as Vit C, with the control showing the highest concentration. It can be seen from the results that the lycopene content decreased significantly from 4.42 mg/100g in the control to 0.40 mg/100g in T4. The control showed the highest lycopene concentration, while T1, T2, T3, and T4 exhibited progressively lower levels. The decrease in lycopene content with increasing whey concentration reflects the dilution effect as the proportion of lycopene-rich tomato decreases. The βC levels showed a consistent decline from the control to T4, again correlating with the tomato content in each formulation as banana content was kept same in all formulations. The control had the highest βC (0.49 mg/100g), with significant reductions observed as the tomato content decreased in the formulations (Figure 29). This trend indicates that as whey concentration increases, the βC, primarily derived from the tomato component, decreases due to reduced tomato content. The TPC in the WMB mixes ranged from 0.14 mg GAE/g in T4 to 0.31 mg GAE/g in T1 and the control had a TPC of 0.53 mg GAE/g (Figure 30). With the p-value significant, the general trend showed that the higher content of tomato in T1 contributes to the elevated phenolic content compared to others. The trend reflects that the TPC increases with the proportion of tomato in the formulations.

Table 12. Vit C and Lycopene of WMB mix

Sample	Vit C (mg/100g)	Lycopene (mg/100g)
C	11.35±0.11^{e}	4.42±0.22^{c}
T1	6.13±0.09^{d}	2.28±0.41^{b}
T2	3.82±0.09^{c}	1.65±0.66^{b}
T3	2.73±0.16^{b}	0.58±0.11^{a}
T4	1.56±0.14^{a}	0.40±0.17^{a}
P value	**	**

All values are expressed in Mean±SD. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4. Values with same letters within a column are not significantly different by DMRT ($p < 0.05$). '**' indicates P value <0.001 which is highly significant at 1% level.

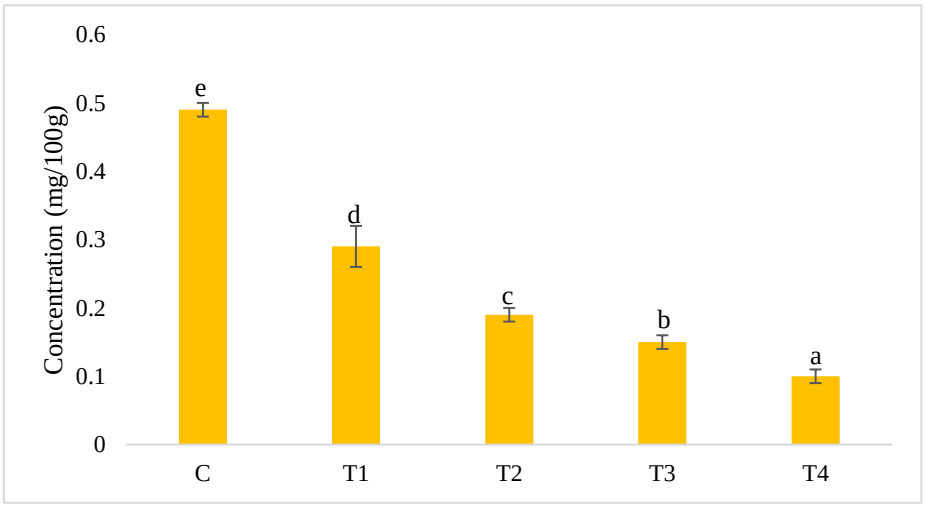


Figure 29. βC content of WMB mixes

All values are expressed in Mean±SD. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4. Bars with different letters are significant at $p < 0.05$.

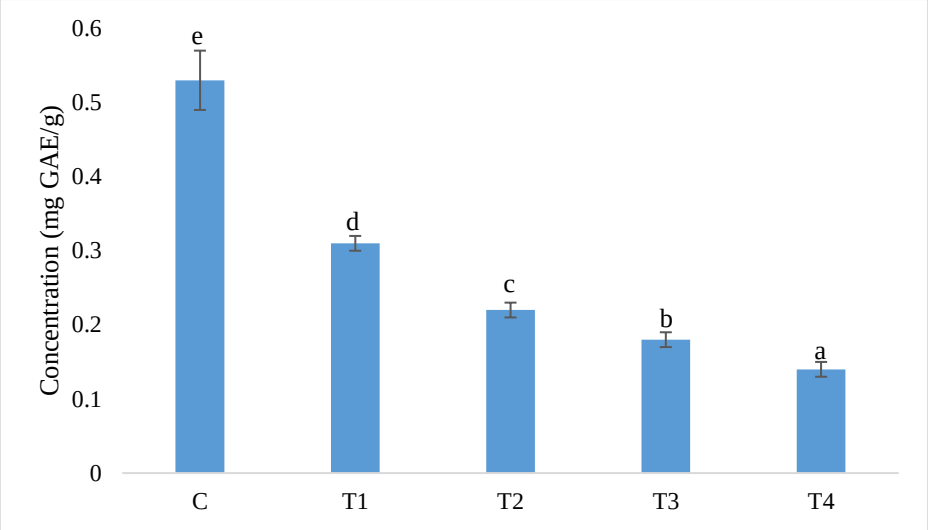


Figure 30. TPC content of WMB mixes

All values are expressed in Mean±SD. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4. Bars with different letters are significant at $p < 0.05$.

5.1.3.2.2 Antioxidant assays

5.1.3.2.2.1 Total Antioxidant Capacity

The TAC was found to be high in the T1 formulation i.e., 322.97 µM Trolox/100g followed by T2 with 219 µM Trolox/100g, T3 with 181 µM Trolox/100g, and T4 with 156 µM Trolox/100g (Figure 31). Interestingly, the TAC values do not strictly follow the trends observed in other parameters. T1 showed a higher TAC value than the control, despite having lower levels of Vit C, lycopene, βC, and TPC than control. This suggests that while tomato contributes significantly to antioxidant capacity, the whey component in T1 may also play a crucial role in enhancing the overall TAC. Whey is known for its antioxidant properties, possibly due to the presence of bioactive peptides and other compounds that can scavenge free radicals. The significant difference in TAC values, particularly the higher TAC in T1 compared to the control, underscores the synergistic effect of whey and tomato in boosting antioxidant capacity in the WMB mixes. The p-value is highly significant (**), indicating that the differences in TAC among the formulations are statistically significant.

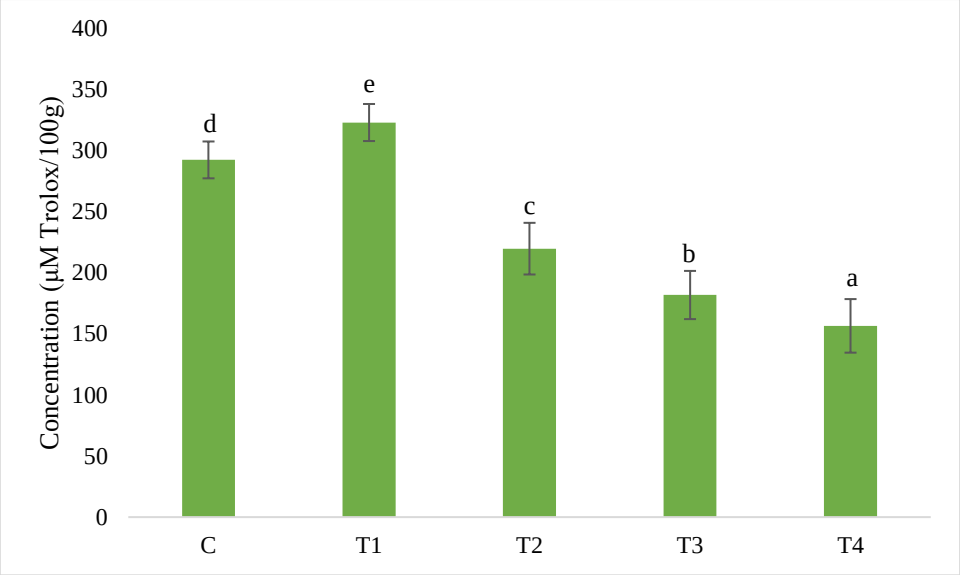


Figure 31. TAC of WMB mixes

All values are expressed in Mean±SD. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4. Bars with different letters are significant at $p < 0.05$.

5.1.3.2.2.2 DPPH assay

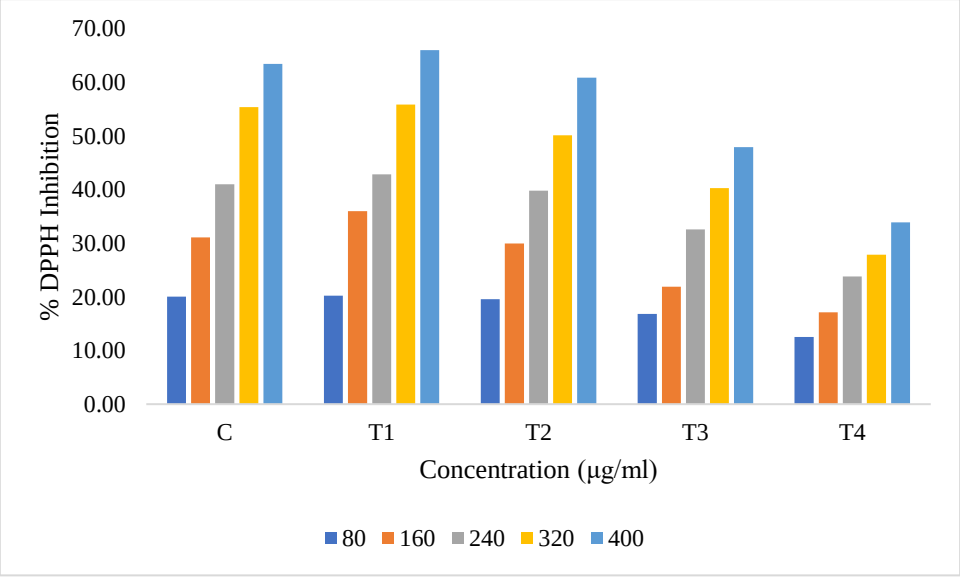


Figure 32. DPPH assay of WMB mixes

All values are expressed in Mean±SD. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4.

The DPPH assay results presented in Figure 32 provide insights into the antioxidant activity of different formulations of WMB mixes at various concentrations (80, 160, 240, 320, and 400 µg/ml). The percentage inhibition values reflect the ability of each formulation to scavenge free radicals, indicating their antioxidant potential. At the lowest concentration of 80 µg/ml, the percentage inhibition values were relatively low for all formulations, with T1 exhibiting the highest inhibition (20.20%) and T4 the lowest (12.55%). This trend continues at higher concentrations, where T1 consistently showed the highest inhibition among the test formulations, reaching 65.98% at 400 µg/ml, surpassing the control which has 63.42%. T2 also performed well, though slightly lower than T1, with 60.85% inhibition at 400 µg/ml.

As the concentration increases, the percentage inhibition values for all formulations improved, indicating a dose-dependent relationship. Notably, T3 and T4 showed lower inhibition values across all concentrations compared to T1 and T2. At the highest concentration of 400 µg/ml, T3 and T4 exhibit inhibition percentages of 47.89% and 33.85%, respectively. The control, with the highest tomato content, showed strong antioxidant activity, particularly at higher concentrations, where it reaches 63.42% inhibition at 400 µg/ml. This suggests that the high tomato content contributes significantly to the antioxidant potential of the control mix. In summary, T1 demonstrated the highest antioxidant activity among the test formulations across all concentrations, likely due to the balanced inclusion of whey and tomato, which provides a synergistic effect.

Table 13 presents the IC_{50} values for the DPPH radical scavenging activity of different WMB mixes. The IC_{50} value represents the concentration of the sample required to inhibit 50% of the DPPH radicals, with lower IC_{50} values indicating higher antioxidant activity. The control sample had an IC_{50} value of 302.22±5.31 µg/mL, demonstrating a relatively strong antioxidant capacity. Among the test formulations, T1 exhibited the lowest IC_{50} value at 279.92±7.35 µg/mL, indicating that T1 has the highest antioxidant activity. This superior performance is

likely due to the balanced presence of whey and tomato, which provides a synergistic effect on antioxidant properties. T2 had a slightly higher IC_{50} value of 320.45±9.17 μg/mL, followed by T3 and T4 (427.20±7.17 μg/mL and 616.95±5.38 μg/mL). These higher values indicate much lower antioxidant activities for these formulations.

Table 13. DPPH IC_{50} values of WMB mixes

Samples	DPPH activity IC_{50} (μg mL^{-1})
C	302.22±5.31
T1	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. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4.

5.1.3.2.2.3 Correlation between various bioactive components

The correlation analysis in Table 14 showed a strong positive and statistically significant interrelationship between bioactive components, such as Vit C, lycopene, βC, TPC, and TAC in WMB mixes. The correlation coefficients indicate the strength and direction of the linear relationship between these variables, with "**" denoting significance at the 1% level. All the components except protein showed a strong positive correlation with each other indicating that there is a corresponding increase in component according to their respective increase of proportion. Protein showed very weak and statistically non-significant correlations with all other components except TAC. These negative correlations, albeit weak, suggest that while protein (primarily from whey) is a significant contributor to TAC, it does not necessarily correlate with the levels of these specific bioactive compounds. The positive correlation between protein content and TAC is particularly noteworthy, as it underscores the role of WP in enhancing the antioxidant properties of the WMB mixes in which plant-based compounds work synergistically to enhance the antioxidant properties of the WMB mixes.

Table 14. Correlation between bioactive components

Parameters	Vit C	Lycopene	βC	TPC	TAC	Protein
Vit C	1	0.973**	0.998**	0.994**	0.996**	-0.090
Lycopene	-	1	0.967**	0.973**	0.998**	-0.067
βC	-	-	1	0.996**	0.996**	-0.101
TPC	-	-	-	1	0.994**	-0.118
TAC	-	-	-	-	1	0.997**
Protein	-	-	-	-	-	1

** denotes significance at 1% level, * denotes significance at 5% level

5.1.3.2.2.4 Effect of bioactive components on TAC by multiple regression analysis

The multiple regression analysis is a statistical technique that analyses the relationship between two or more variables and uses the information to estimate the value of the dependent variables. It is basically explaining or predicting a single dependent variable from two or more independent variables. The current analysis employed TAC as the dependent variable and various bioactive components such as Vit C, lycopene, βC, TPC and protein content as the independent variables (Table 15). The purpose of this analysis was to quantitatively assess the collective and individual impact of these bioactive components on the TAC of WMB mixes and to determine the extent to which each bioactive component independently influences the TAC which is crucial for identifying which components are the most significant contributors as well as to understand how each component contributes to the antioxidant properties of the WMB mixes when other factors are held constant.

5.1.3.3 Physicochemical property analyses

The physicochemical property analyses of WMB mixes, as presented in Table 16, reveals significant variations across different formulations (T1, T2, T3, and T4). The water activity of the formulations showed a clear increasing trend from T1 to T4, with T1 having the lowest value and T4 the highest. The values ranged from 0.25±0.01 in T1 to 0.41±0.01 in T4, indicating that higher whey concentrations resulted in higher water activity. The differences

between all formulations are statistically significant ($p < 0.05$), as indicated by different superscript letters. Total solids content decreases progressively from T1 (96.13±0.09%) to T4 (95.51±0.10%), with each formulation showing a statistically significant difference from the others ($p < 0.05$). This trend suggests that increasing whey concentration slightly reduces the total solids in the mix. Similarly, TSS measured in °Brix also decrease significantly from T1 (11.43±0.40) to T4 (9.27±0.21), with all formulations showing significant differences ($p < 0.05$).

The pH values of the formulations increased from T1 (4.68±0.01) to T4 (5.13±0.01), with each formulation significantly different from the others ($p < 0.05$). This increase in pH correlates with the decreasing acidity observed in the titrable acidity values, which decreased from T1 (0.27±0.01%) to T4 (0.22±0.01%). Both parameters showed significant differences across all formulations ($p < 0.05$). Viscosity also showed a decreasing trend from T1 (10±0.01 cP) to T4 (7.00±1.15 cP), with significant differences between each formulation ($p < 0.05$). The parameter a* takes positive values for reddish colours and negative values for the greenish ones, whereas b* takes positive values for yellowish colours and negative values for the bluish ones. L* is an approximate measurement of luminosity, which is the property according to which each colour can be considered as equivalent to a member of the greyscale, between black and white (145). The L* values range from 69.34±0.5 in T2 to 70.81±0.01 in T4, with T4 being significantly different from the others ($p < 0.05$). The a* values, indicating redness, decrease significantly from T1 (8.67±0.22) to T4 (4.22±0.01), showing a clear reduction in red hue with higher whey content. Similarly, the b* values, indicating yellowness, decrease from T1 (22.75±0.39) to T4 (15.01±0.02), with all differences being statistically significant ($p < 0.05$).

Table 15. Effect of bioactive components on TAC by multiple regression analysis

Variables	Unstandardized coefficient (B)	SE of B	Standardized coefficient (Beta)	t value	P value
Constant	-1.497	0.776	-	1.928	0.095
Vit C	0.793	0.244	0.273	3.245	0.005**
Lycopene	0.139	0.024	0.614	5.737	0.001**
βC	0.167	0.031	0.602	5.432	0.001**
TPC	1.235	0.299	0.443	4.133	0.004**
Protein	0.148	0.016	0.761	9.049	0.000**

** Denotes significance at 1% level

Table 16. Physicochemical property analysis of WMB mixes

Parameters	C	T1	T2	T3	T4	P value
Water Activity (aW)	0.42 ± 0.01^{e}	0.25 ± 0.01^{a}	0.30 ± 0.01^{b}	0.36 ± 0.01^{c}	0.41 ± 0.01^{d}	**
Total Solids (%)	95.65 ± 0.11^{ab}	96.13 ± 0.09^{d}	95.86 ± 0.09^{c}	95.68 ± 0.04^{b}	95.51 ± 0.10^{a}	**
TSS (°Brix)	4.52 ± 0.02^{a}	11.43 ± 0.40^{e}	10.63 ± 0.15^{d}	10.23 ± 0.06^{c}	9.27 ± 0.21^{b}	**
pH	4.22 ± 0.03^{a}	4.68 ± 0.01^{b}	4.85 ± 0.01^{c}	5.05 ± 0.01^{d}	5.13 ± 0.01^{e}	**
Titrable Acidity (%)	0.33 ± 0.02^{c}	0.27 ± 0.01^{b}	0.24 ± 0.01^{a}	0.23 ± 0.01^{a}	0.22 ± 0.01^{a}	**
Viscosity (cP)	13.33 ± 1.53^{d}	10 ± 0.01^{c}	8.67 ± 1.15^{bc}	8.00 ± 0.01^{ab}	7.00 ± 1.15^{a}	**
L*	34.05 ± 1.53^{a}	69.77 ± 0.41^{bc}	69.34 ± 0.5^{b}	69.63 ± 0.18^{bc}	70.81 ± 0.01^{c}	**
a*	12.08 ± 1.62^{d}	8.67 ± 0.22^{c}	7.98 ± 0.09^{c}	5.84 ± 0.11^{b}	4.22 ± 0.01^{a}	**
b*	31.27 ± 1.25^{d}	22.75 ± 0.39^{c}	22.78 ± 0.03^{c}	16.50 ± 0.19^{b}	15.01 ± 0.02^{a}	**

All values are expressed in Mean±SD. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4. Values with same letters within a row are not significantly different by DMRT ($p < 0.05$). '**' indicates P value <0.001 which is highly significant at 1% level.

5.1.3.4 Functional property analyses

Table 17. Functional properties of WMB mix

Sample	Solubility (%)	Wettability (s)	Dispersibility (%)	WAI (%)	WSI (%)
C	65..5±0.70^{a}	21.50±2.12^{c}	73.50±2.12^{a}	1.90±0.14^{b}	0.82±0.02^{ab}
T1	79.50±0.70^{e}	10.25±0.35^{a}	83.50±2.12^{c}	2.40±0.14^{c}	0.87±0.01^{c}
T2	77.00±1.41^{d}	11.25±0.35^{a}	80.25±0.35^{bc}	2.20±0.07^{c}	0.84±0.01^{bc}
T3	74.50±0.70^{c}	12.50±0.70^{a}	77.5±2.12^{ab}	1.75±0.07^{b}	0.83±0.01^{abc}
T4	70.5±0.70^{b}	15.50±0.70^{b}	76.50±2.12^{ab}	1.26±0.02^{a}	0.80±0.01^{a}
P value	**	**	*	**	*

All values are expressed in Mean±SD. C-control; T1- Test formulation 1; T2- Test formulation 2; T3- Test formulation 3; T4- Test formulation 4. Values with same letters within a column are not significantly different by DMRT ($p < 0.05$). '**' indicates P value <0.001 which is highly significant at 1% level, '*" indicates P value between 0.011 to 0.050 which is significant at 5% level.

The results presented in Table 17 describe the functional properties of the WMB mixes, particularly solubility, wettability, dispersibility, WAI, and WSI. T1 showed the highest solubility, followed by T2, T3, and T4, with the control (C) showing the lowest value. T1 exhibited the lowest wettability time (10.25 s), indicating faster rehydration, while the control had the longest wettability (21.50 s). Besides, T1 showed the highest dispersibility (83.50%), indicating better rehydration properties, with the control exhibiting the lowest dispersibility (73.50%). T1 and T2 had higher WAI values than the control, with T4 showing the lowest. WSI values were also highest for T1, followed by T2, T3 and T4.

5.1.3.5 Rheological behaviour of WMB

Table 18 presents the rheological behaviour of WMB mixes under different conditions and highlights the pseudoplastic nature of the formulations. The consistency coefficient (k) increases with higher concentrations of whey (T4 showing the highest at both temperatures). This reflects the increased viscosity and greater resistance to flow due to WPs. The flow behaviour index (n) values are less than 1, indicating pseudoplastic behaviour, where viscosity decreases with increasing shear rate, typical of non-Newtonian fluids. At 10°C, all formulations exhibited higher k values compared to 25°C, indicating increased viscosity at lower temperatures. For example, T4's k value increased from 0.193 (25°C) to 0.221 (10°C), reflecting temperature's influence on protein and polysaccharide interactions in whey and tomato (146).

Table 18. Rheological behaviour of WMB

Test Formulation	Temperature (^oC)	Consistency coefficient-k	Flow behaviour index-n	Fit (R^2)
T1	25	0.054	0.415	0.722
T2		0.070	0.334	0.702
T3		0.113	0.273	0.607
T4		0.193	0.232	0.449
T1	10	0.057	0.421	0.998
T2		0.105	0.315	0.993
T3		0.190	0.321	0.996
T4		0.221	0.544	0.990

T1- Test formulation 1; T2- Test formulation 2; T3-Test formulation 3; T4- Test formulation 4

5.1.3.6 Sensory evaluation of WMBs

The sensory evaluation of WMB formulations (C, T1, T2, T3, and T4) revealed significant differences among the samples (Table 19). T1 consistently received the highest scores across

most sensory attributes, particularly for taste (7.45±1.36), appearance (7.50±0.89), and overall acceptability (7.42±0.36) among other formulations and control. Its combination of moderate whey (45%), tomato, along with banana pulp provided a balanced sensory experience, leading to better overall scores. The control (C) formulation, primarily tomato-based, had a good score for appearance (7.00±1.17) but performed poorly in texture (5.55±1.73) and overall acceptability (5.35±1.56), due to the absence of whey, which influences mouthfeel and creaminess. As the whey content increased (from T2 to T4), the sensory attributes, particularly appearance, taste, and overall acceptability, showed a downward trend. T4 (with 85% whey) had the lowest scores, particularly for appearance (5.45±1.19) and taste (5.25±1.07).

Statistical analysis using DMRT ($p < 0.05$) showed significant differences in appearance, texture, taste, and overall acceptability across the formulations. T1 significantly outperformed the other samples, with all parameters showing statistical significance ($p < 0.05$) when compared with the control, indicating that the combination of ingredients, particularly the 45% whey in T1, provided an optimal sensory experience. T4, on the other hand, had the lowest scores across the board, with its high whey content likely causing off-putting sensory qualities.

Table 19. Sensory evaluation of formulations

Attributes	C	T1	T2	T3	T4
Appearance and colour	7.00 ± 1.17^{bc}	7.50 ± 0.89^{c}	7.15 ± 1.04^{c}	6.40 ± 1.27^{b}	5.45 ± 1.19^{a}
Flavour	5.95±1.82	6.60±1.35	6.60±1.35	6.30±1.22	5.95±1.09
Consistency/ texture	5.55 ± 1.73^{a}	7.00 ± 1.30^{c}	7.10 ± 0.80^{c}	6.80 ± 1.43^{bc}	6.05 ± 1.23^{ab}
Taste	5.30 ± 1.84^{a}	7.45 ± 1.36^{b}	6.25 ± 1.45^{ab}	6.20 ± 1.61^{ab}	5.25 ± 1.07^{a}
Mouthfeel/ after taste	5.25±1.74	6.15±1.63	6.05±1.53	5.85±1.56	5.30±0.98
Overall acceptability	5.35 ± 1.56^{a}	7.42 ± 0.36^{c}	6.40 ± 1.43^{b}	6.15 ± 1.27^{ab}	5.70 ± 0.98^{ab}

All values are expressed in Mean±SD. T1- Test formulation 1; T2- Test formulation 2; T3-Test formulation 3; T4- Test formulation 4. Values with same letters within a row are not significantly different by DMRT ($p < 0.05$).

5.1.3.7 Selection of final optimized test formulation

Based on the comprehensive analysis of proximate composition, nutritional content, physicochemical, functional, sensory properties, bioactive compounds profile, and antioxidant assays, it was determined that the T1 formulation demonstrated the most favourable overall profile for further evaluation. Consequently, the T1 formulation was selected for subsequent assays, while the control (C), T2, T3, and T4 formulations were excluded from further testing except C employed for cell line studies. This decision reflects T1's superior performance across various assessment criteria, highlighting its potential as the optimal candidate for further development and study. It delivers a high protein content, improved rehydration properties, and enhanced sensory appeal while retaining adequate antioxidant activity. This holistic combination of nutritional and functional benefits makes T1 the most suitable formulation for beverage applications specific for cancer formulations. The following points laid down the justification for the selection of T1 over other formulations for the further assays.

1) T1 provides an optimal balance between protein (whey) and other proximate nutrients from tomato, banana, SOC, and FOC. Its moderate whey content (45%) ensures a high protein concentration while maintaining a good balance of other nutrients from the fruit pulps and oil cakes.
2) T1 showed superior functional properties, including solubility (79.50±0.70%), wettability, and dispersibility (83.50±2.12%), which enhance its rehydration characteristics, making it more suitable for instant beverage applications. These values are significantly better than the control (C), which has lower solubility (65.50±0.70%) and poorer dispersibility (73.50±2.12%) due to the absence of WP.
3) T1 significantly outperformed other formulations in sensory evaluations, particularly in taste (7.45±1.36), texture (7.00±1.30), and overall acceptability (7.42±0.36). This is attributed to the balanced whey concentration that improves mouthfeel, creaminess, and palatability. The control and higher whey formulations (T3 and T4) scored lower due to

textural, as well as sensorial issues as the control lacked the creamy texture provided by WP.

4) T1 maintains a favourable balance of bioactive compounds from tomato, banana pulp (Vit C, lycopene, βC, TPC), contributing to its higher antioxidant capacity compared to T3 and T4. While the control has higher lycopene content due to its 88% tomato composition, the inclusion of whey in T1 allows it to retain adequate antioxidant potential mostly by its antioxidant bioactive peptides without compromising on sensory properties, which is crucial for consumer acceptability.

5.1.3.8 Mineral profile of T1 WMB mix

The mineral analysis was performed on T1 WMB mix and the results are represented in the Figure 33 and Figure 34. The major minerals in T1 WMB mix, particularly potassium, magnesium, calcium, and phosphorus, contribute to its antioxidant and anticancer activities by supporting enzymatic functions, maintaining cellular homeostasis, and modulating oxidative stress. The analysis of trace minerals in the T1 WMB revealed significant levels of zinc (Zn), manganese (Mn), and copper (Cu), which contribute to the functional and health-promoting properties of the product.

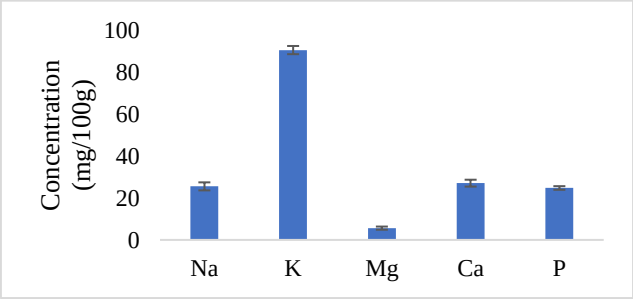


Figure 33. Major minerals in T1 WMB mix

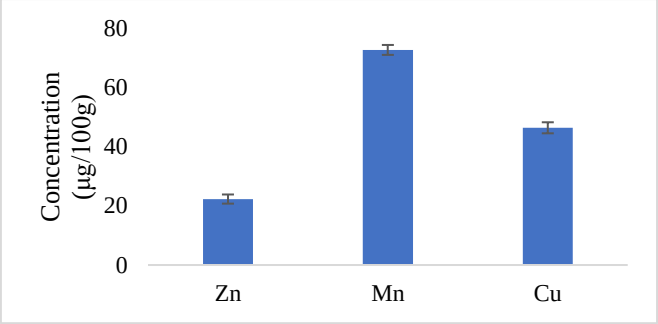


Figure 34. Trace minerals in T1 WMB mix

5.1.3.9 Protein quality analyses of T1 WMB mix

Some popular techniques for figuring out the protein quality of food samples are the information pertaining to their amino acid content, and IVPD. These analyses were carried out for T1 WMB mix and the results are depicted in below sections.

5.1.3.9.1 Amino acid analysis of T1 WMB mix

The amino acid analyses were carried out for T1 WMB mix and results are depicted in Table 20 and the UHPLC chromatogram is represented in Appendix IX. With the exception of tryptophan, which was eliminated during hydrolysis with HCL, the amino acid profile revealed that T1 mix contained all of the EAAs.

5.1.3.9.2 IVPD assay of T1 WMB mix

The *In Vitro* Protein Digestibility (IVPD) assay is a widely used method to estimate the extent of protein digestion and absorption in the gastrointestinal tract. It provides an indirect measure of the bioavailability and quality of the protein. The IVPD value of T1 WMB mix, which stands at 86.83%, is considered quite high and reflects the excellent digestibility of the WP included in the formulation and is on par when compared with the reference standard of casein with the IVPD value of 90.90%.

Table 20. Amino acid profiling of T1 WMB mix

SL. No	Amino acids	Retention Time (min)	Area	Concentration (mg/g)
1	Aspartic Acid	3.37	1827577	0.1936
2	Glutamic Acid	3.78	805881	0.1919
3	Serine	4.45	2567600	0.1997
4	Histidine	9.02	5427317	0.4649
5	Glycine	10.89	45153	0.0068
6	Threonine	12.28	2226684	0.1292
7	Arginine	12.67	1942379	0.1984
8	Alanine	13.76	1648127	0.0730
9	Tyrosine	16.41	8757209	0.8286
10	Cystine	20.23	579591	0.0743
11	Valine	26.89	6620833	0.3603
12	Methionine	27.59	130812	0.0091
13	Phenylalanine	32.11	1253436	0.1001
14	Isoleucine	32.7	3565058	0.2006
15	Leucine	34.91	8610220	0.4888
16	Lysine	36.08	128445	0.0232
17	Proline	39.47	21310	0.0216

5.1.3.10 Microbial evaluation of T1 WMB mix

The figure 35 appears to present the microbial evaluation of T1 WMB mix under different storage conditions, focusing on viable count (CFU/g). The analysis includes data at two temperatures: ambient temperature (25 ± 5°C) and refrigerated temperature (4 ± 1°C). The samples were stored in three types of packaging materials: glass bottles, plastic pouches, and metallic pouches, with evaluations at 0 and 90 days. The microbial growth is tested on different

culture media, namely Nutrient Agar (NA), Eosin Methylene Blue Agar (EMB), Salmonella-Shigella Agar (SS), and Rose Bengal Agar (RB).

Sample	Viable count (CFU/g)											
	Ambient Temperature (25±5°C)						Refrigerated Temperature (4±1°C)					
	Glass bottle		Plastic pouch		Metallic pouch		Glass bottle		Plastic pouch		Metallic pouch	
	0	90	0	90	0	90	0	90	0	90	0	90
NA	Absent	108x 10^3	Absent	67x 10^3	Absent	58x 10^3	Absent	57x 10^3	Absent	49 x 10^3	Absent	34 x 10^3
EMB	Absent	<50	<50	<50	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
SS	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
RB	Absent	<50	Absent	<50	Absent	<50	Absent	<50	Absent	Absent	Absent	Absent

Figure 35. Microbial evaluation of T1 WMB mix

5.1.3.11 Shelf-life studies

5.1.3.11.1 Effect of storage on physicochemical parameters of the T1 WMB mix

Table 21 Effect of storage on physicochemical parameters of the T1 WMB mix

Days	0	45	90
TSS	11.43±0.40	12.37±2.12	13.87±1.21
Acidity	0.27±0.01	0.43±0.06	0.63±0.04
pH	4.60±0.01	4.54±0.04	4.40±0.023

There is a noticeable increase in TSS over the storage period. At day 0, the TSS value starts at 11.43%. By day 45, TSS increases to 12.37%, and by day 90, it reaches 13.87%. The gradual rise in TSS could be due to moisture loss or concentration of solutes as the product undergoes storage. This increase in TSS may affect the taste, sweetness, and overall sensory characteristics of the beverage, potentially making it more concentrated over time.

Acidity shows a significant rise from 0.27% at day 0 to 0.63% by day 90. This increase indicates that the beverage becomes more acidic as it is stored. The rising acidity may be attributed to ongoing fermentation processes or enzymatic activities in the WMB mix, which can lead to the production of organic acids. Higher acidity levels can affect flavour, shelf life, and microbial stability, potentially lowering the pH and making the product less favourable to microbial growth.

The pH of the WMB mix decreases slightly over time, from 4.60 on day 0 to 4.40 at day 90. This decrease is consistent with the observed rise in acidity. The drop in pH indicates that the product is becoming more acidic as storage progresses. A lower pH can improve the product's microbial safety, as many pathogens do not thrive in acidic environments. However, a decline in pH may also impact the sensory qualities, such as flavour, making the beverage taste tangier or sourer over time.

5.1.3.11.2 Effect of storage on sensory parameters of the T1 WMB mix

The sensory analysis of the T1 WMB mix provides crucial insight into how the product's appeal changes over time. Evaluating the sensory attributes such as appearance and colour, flavour, consistency/texture, taste, mouthfeel/aftertaste, and overall acceptability allows for a better understanding of consumer perceptions during the product's storage period. Sensory changes are especially important when determining the shelf life, as these parameters directly influence consumer preferences and purchasing decisions.

Table 22. Effect of storage on sensory parameters of the T1 WMB mix

Day	0	45	90
Appearance and colour	7.50±0.89	6.40±1.89	6.00±2.35
Flavour	6.60±1.35	6.90±1.74	6.80±1.20
Consistency/Texture	7.00±1.30	6.32±1.26	6.15±1.76
Taste	7.45±1.36	6.05±1.13	6.00±1.73
Mouthfeel/after taste	6.15±1.63	6.30±1.37	6.00±1.14
Overall acceptability	7.42±0.36	7.00±41	6.60±1.35

5.1.3.12 Anticancer activity assays

5.1.3.12.1 Determination of cytotoxicity of individual ingredient samples

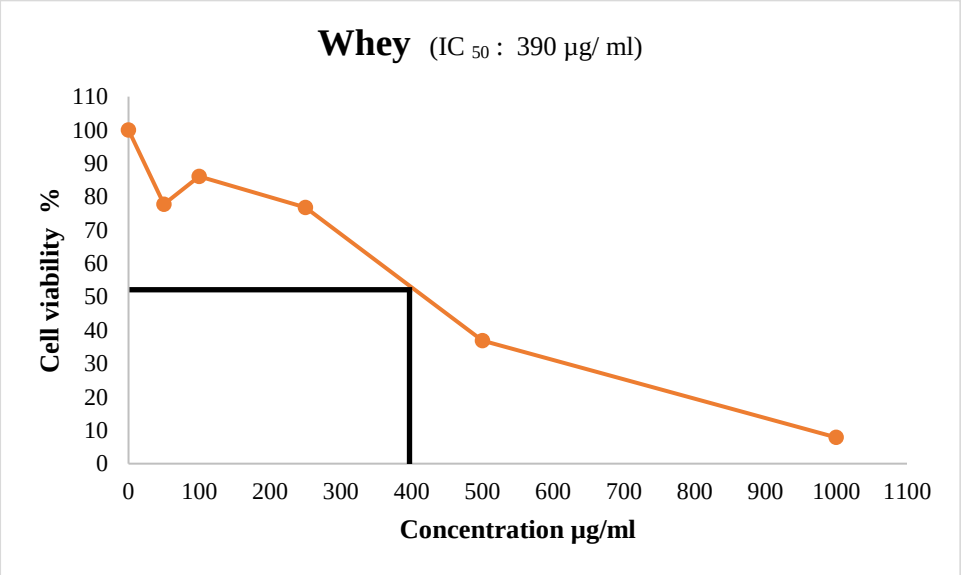


Figure 36. *In vitro* cytotoxicity of whey against KB OC cells

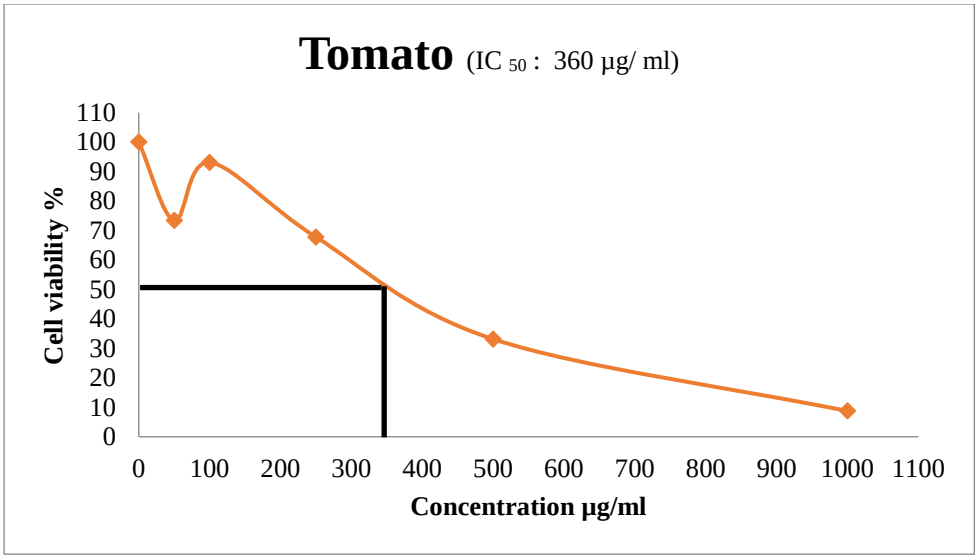


Figure 37. *In vitro* cytotoxicity of tomato against KB OC cells

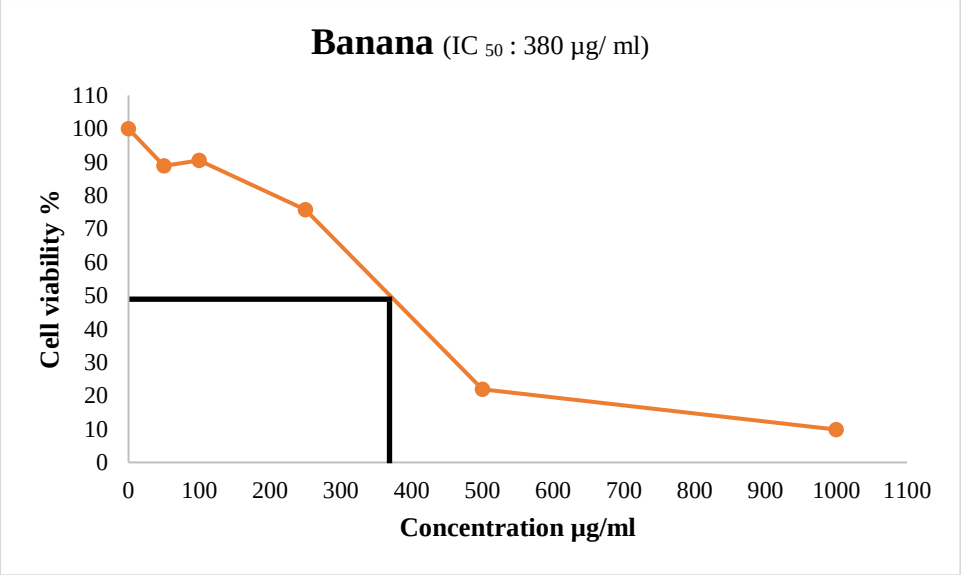


Figure 38. *In vitro* cytotoxicity of banana against KB OC cells

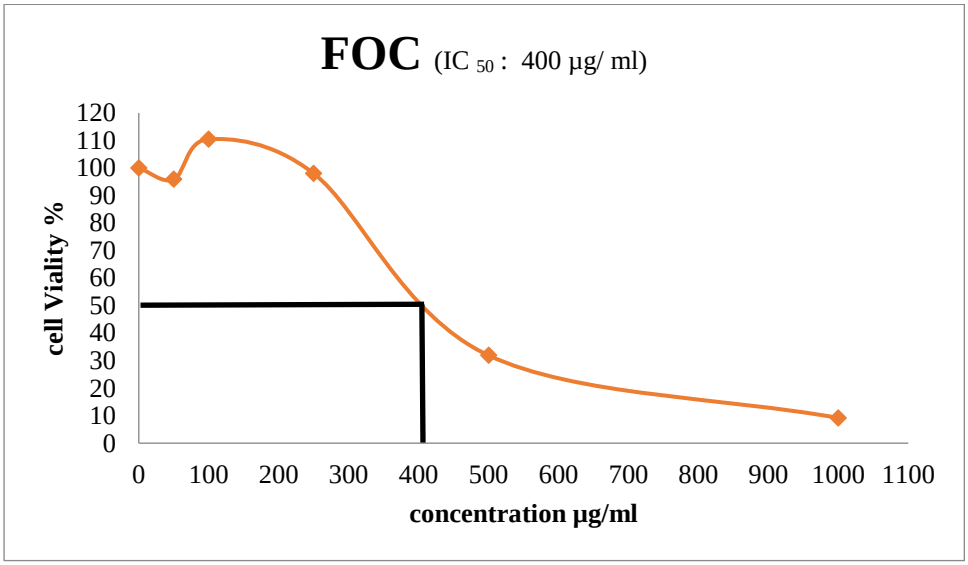


Figure 39. *In vitro* cytotoxicity of FOC against KB OC cells

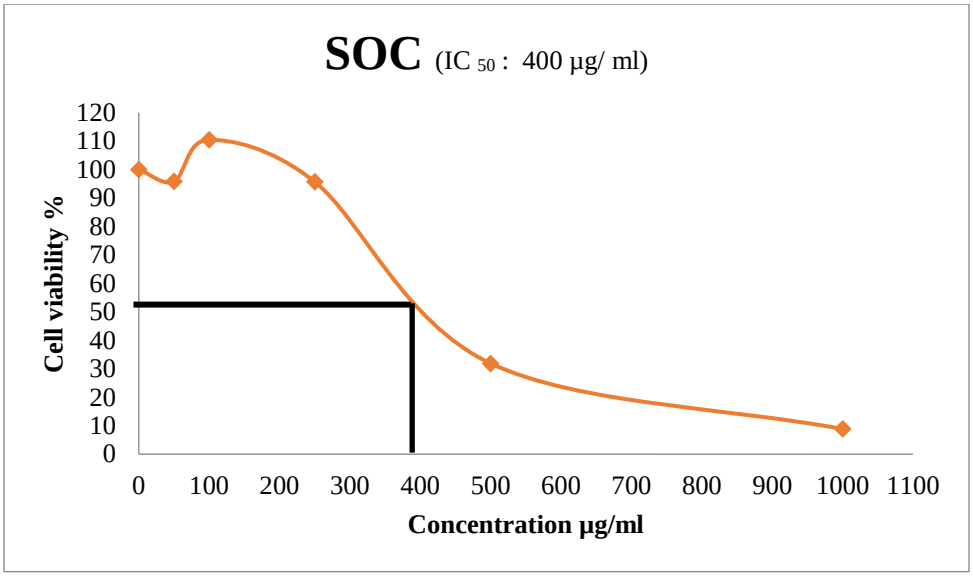


Figure 40. In vitro cytotoxicity of SOC against KB OC cells

5.1.3.12.1 The cytotoxicity of individual ingredients

Determining the anticancer potential of individual ingredient samples were done prior to the investigation of cytotoxicity of T1 WMB mix by MTT assay. The assay was applied for the KB OC cell lines and the detailed description of the results is given hereunder.

5.1.3.12.1.1 *In vitro* cytotoxicity of whey against KB OC cells

The figure 36 indicate a dose-dependent cytotoxicity of whey against KB OC cells, with significant reductions in cell viability at higher concentrations. At lower concentrations (50 and 100 µg/ml), the cell viability is moderately reduced to approximately 77.76% and 86.11%, respectively. However, as the concentration increases to 250 µg/ml, cell viability further declines to 76.82%. Notably, at 500 µg/ml, a substantial drop in viability occurs, with only 36.84% of cells remaining viable. At the highest concentration of 1000 µg/ml, the viability plummets drastically to 7.86%.

5.1.3.12.1.2 *In vitro* cytotoxicity of tomato against KB OC cells

The cytotoxicity of tomato against KB OC cells, as depicted in figure 37, demonstrated a dose-dependent decrease in cell viability. At 50 µg/ml, the cell viability is 73.41%, significantly

decreasing to 8.73% at 1000 µg/ml, indicating strong cytotoxicity suggesting that higher concentrations of tomato extract have potent cytotoxic effects.

5.1.3.12.1.3 *In vitro* cytotoxicity of banana against KB OC cells

The data in figure 38 presents the cytotoxicity of Nendran banana extract against KB OC cells at varying concentrations. At lower concentrations (50 µg/ml and 100 µg/ml), the cell viability is relatively high, at 88.87% and 90.48%, indicating limited cytotoxicity. However, with increasing concentrations, there is a significant drop in cell viability, with 75.69% at 250 µg/ml, 21.89% at 500 µg/ml, and 9.85% at 1000 µg/ml. This dose-dependent cytotoxic response underscores the potential anticancer activity of Nendran banana, particularly at concentrations of 500 µg/ml and above.

5.1.3.12.1.4 *In vitro* cytotoxicity of FOC and SOC against KB OC cells

Figure 39 presents the in vitro cytotoxicity of FOC against KB OC cells across different concentrations. At 50 µg/ml and 250 µg/ml, the cell viability remains relatively high, at 95.87% and 98.00%, respectively. At 100 µg/ml, there is a slight increase in cell viability to 110.48%, which may be attributed to a mild proliferative or protective effect of FOC at this concentration. However, at higher concentrations, the cytotoxic effect becomes significant, with cell viability dropping to 31.88% at 500 µg/ml and to 9.17% at 1000 µg/ml, indicating strong cytotoxic potential of FOC at higher doses.

Figure 40 presents the in vitro cytotoxicity of SOC against KB OC cells across different concentrations. At lower concentrations (50 µg/ml and 100 µg/ml), the cell viability remains high, with a slight increase above 100% at 100 µg/ml. However, at higher concentrations, particularly at 500 µg/ml and 1000 µg/ml, a significant reduction in cell viability is observed, with 31.89% viability at 500 µg/ml and only 8.85% viability at 1000 µg/ml.

5.1.3.12.2 Determination of cytotoxicity of T1 & Doxorubicin against KB OC cells

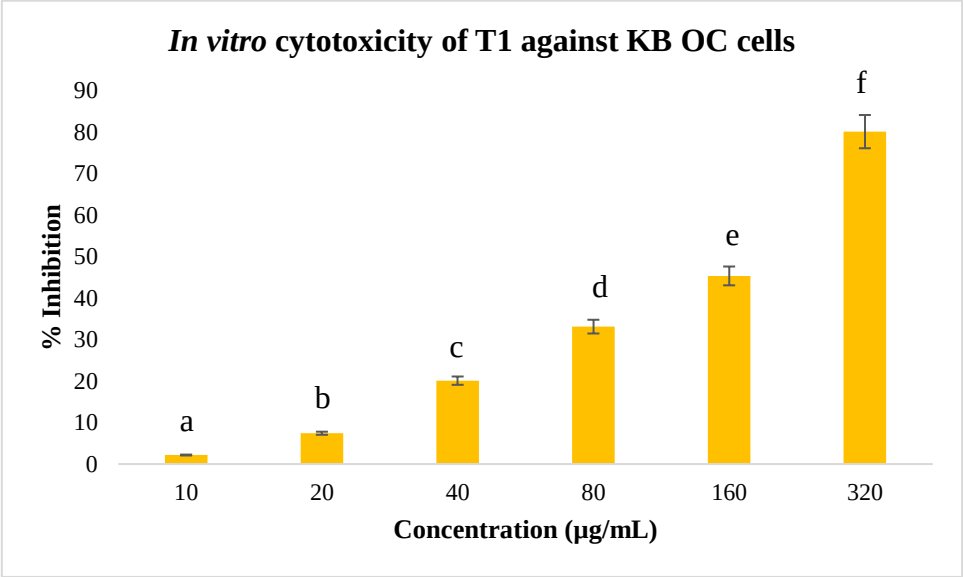


Figure 41. Cytotoxic potential of T1 against KB OC cells

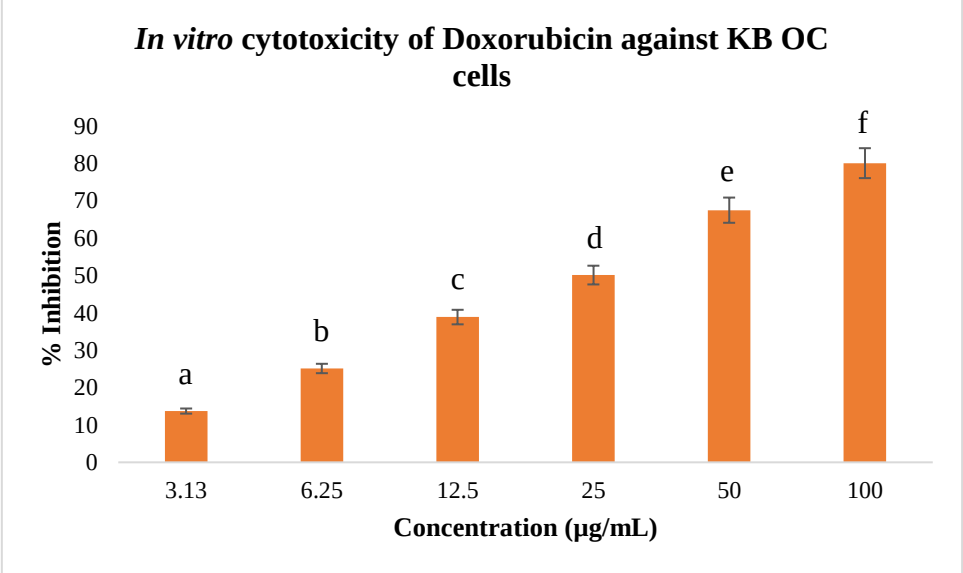


Figure 42. Cytotoxic potential of Doxorubicin against KB OC cells

Each figure is stated for three replicates (n = 3) and bars sharing the same letters are not significantly different ($p \leq 0.05$) conforming to DMRT. The vertical bar indicates the SD.

5.1.3.12.3 Determination of cytotoxicity of T1 & Doxorubicin against normal 3T3-L1 cells

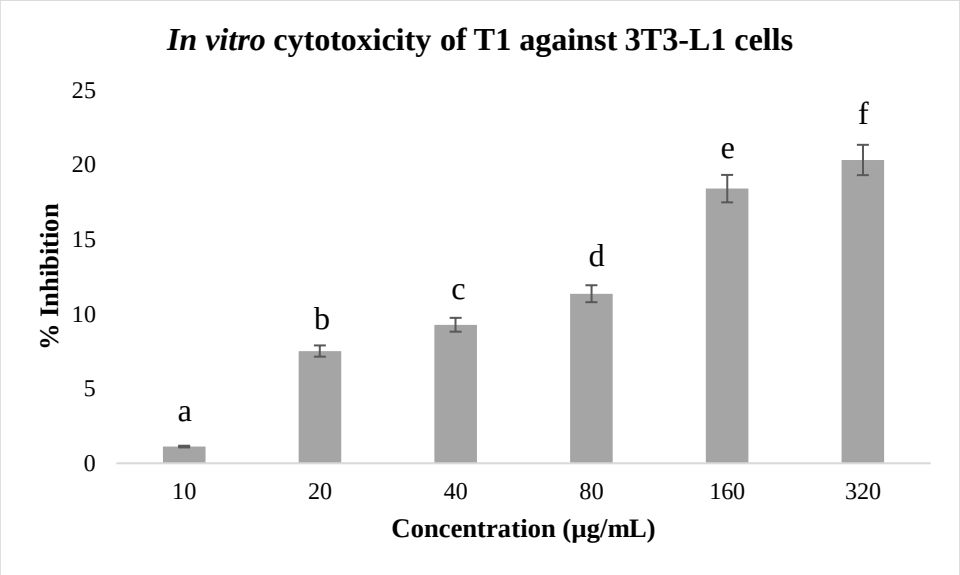


Figure 43. Cytotoxic potential of T1 against 3T3-L1 cells

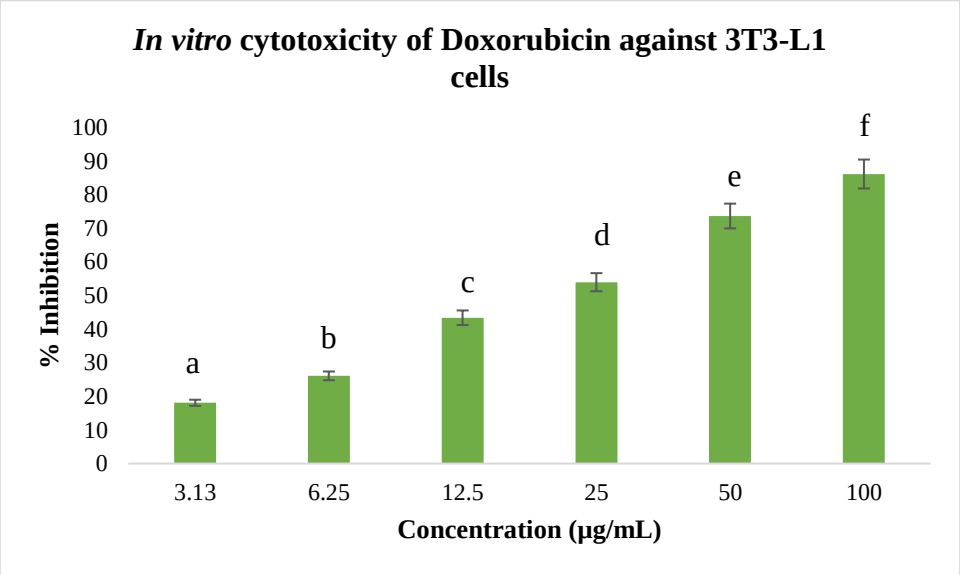


Figure 44. Cytotoxic potential of Doxorubicin against 3T3-L1 cells

Each figure is stated for three replicates (n = 3) and bars sharing the same letters are not significantly different ($p \leq 0.05$) conforming to DMRT. The vertical bar indicates the SD.

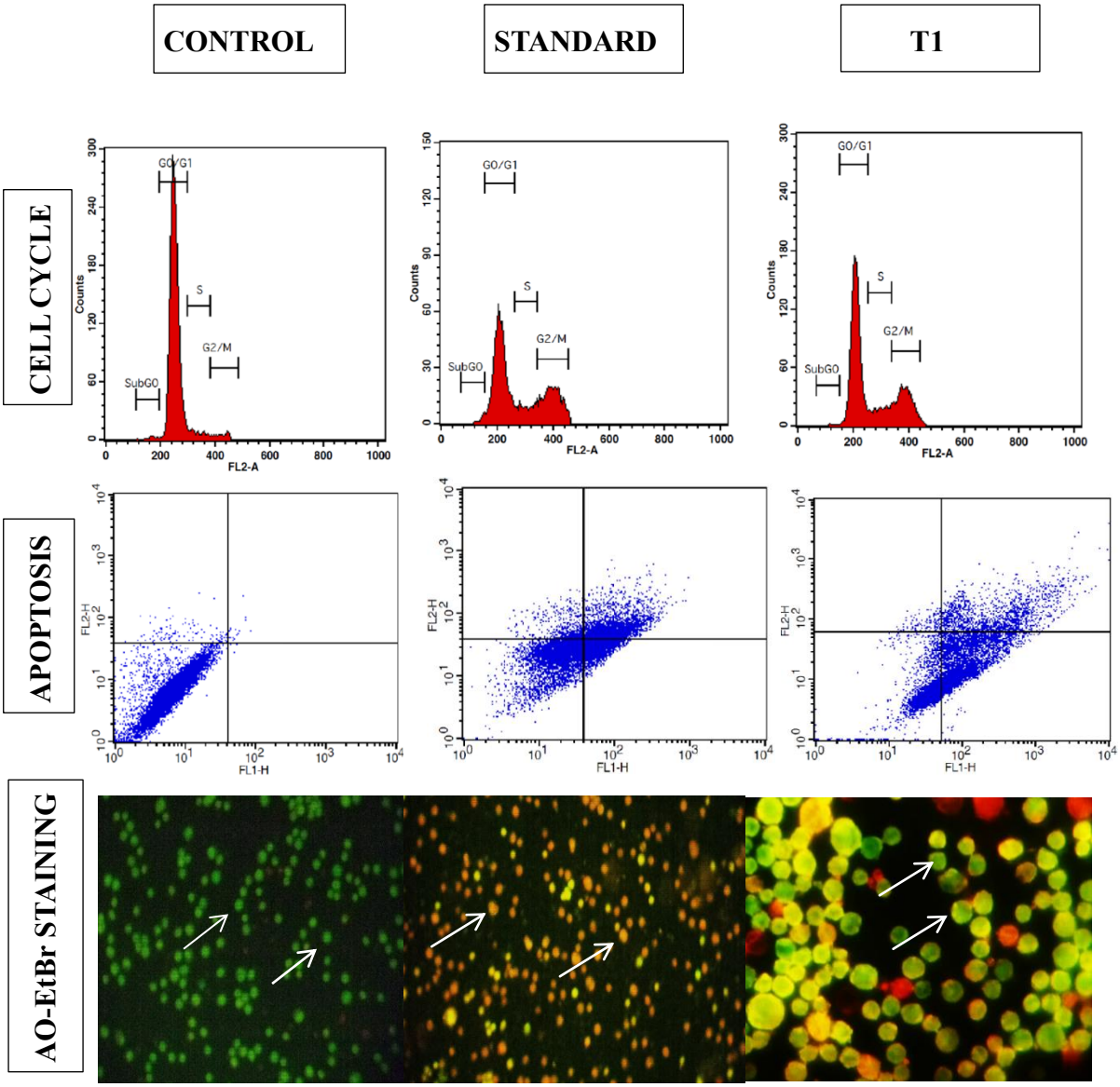


Figure 45. Anticancer potential of T1 against KB OC cells subsequent to various treatments (control – untreated cells, standard-doxorubicin-treated cells)

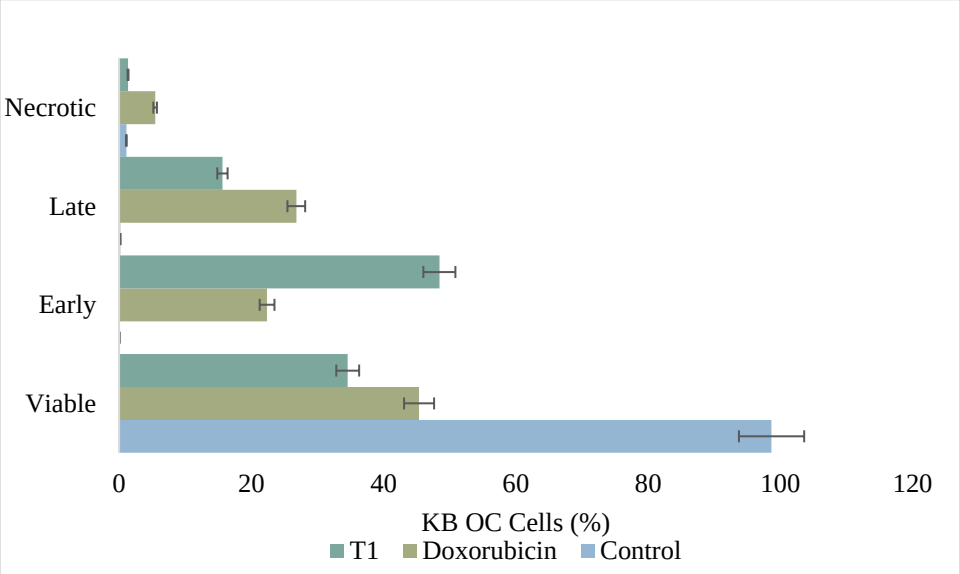


Figure 46. Cell death analysis of T1 against KB Oral Cancer cells

(Each figure is stated for three replicates (n = 3) and the horizontal bar indicates the SD; control – untreated cells)

CHAPTER 6
DISCUSSION

6.0 DISCUSSION

6.1 Quality analysis of ingredients

The results of quality analyses comprising nutritional and physicochemical analyses of whey, tomato, banana, flaxseed, and sesame seed oil cakes are represented in Table 11. The results pertaining to quality aspects of whey showed the characteristics of cheddar whey (sweet whey), and all the basic nutrient parameters like moisture, fat, protein, carbohydrate, and ash content were on par with values described in previous results on this line (51,147–150). The high moisture content necessitates proper handling and processing for storage and utilization and also accounts for the solubility of whey water (150). The fat content aligns with characteristics for cheese whey, while the fairly adequate protein content suggests potential for further protein recovery and utilization. This fat is the component of whey that gives it its foam stability, while the protein part of the whey also gives it the gelling, foaming, and enhanced whipping qualities apart from profound nutritional benefits (150). The ash content indicates the presence of some crucial minerals and gives the product its ionic strength. Carbohydrates (primarily lactose) may contribute to the TSS and might require lactose hydrolysis for some applications. The measured acidity and pH are characteristics of sweet cheese whey, which is in charge of maintaining acid stability. These findings pointed out that slight variations in compositional values could result from the milk source, the conditions under which it is processed, and the milk's chemical makeup itself (17,52).

All the basic nutrient parameters of the Arka Apeksha variety of tomato samples, such as moisture, fat, protein, ash, and carbohydrates, were matched up to the values documented in previous written works (151–153). The TPC determined was found to be higher when compared against findings on Arka Apeksha tomato TPC value of 2.63 mg/100g and on par with results on tomato fruits by Biachi et al. 2023, suggesting a rich profile of phenolic compounds with potential antioxidant properties (154,155). Tomato fruit secondary metabolite

composition (including TPC) is significantly influenced by environmental variables, especially the influence of growing conditions and genetics (156). The high Vit C content indicates a significant source of this essential nutrient, although the observed values were slightly lower when compared to earlier data (20.67 and 26.85 mg/100g) (154,157), which could be due to the higher nutrient absorption and other heritable traits. Lycopene and βC, which are 11- and 9-conjugated carotenes, respectively, are the main pigments found in red tomato fruits (158). Lycopene content, a marker for antioxidant activity and colour, and βC content were fairly high, which underlines the significant profile of these crucial components. These values were within the range reported by researchers in this field (127,154,158–160). In addition, the tomatoes also exhibited a fairly good TSS content, acidity, and pH, which are in accordance with the values reported earlier for Arka Apeksha tomatoes and are also in line with the parameters aimed at by tomato processing industries (127,154,157). Higher nutrient absorption and inherited traits could be the cause of the elevated TSS (157). These findings contribute valuable data on the nutritional profile of Arka Apeksha tomatoes, highlighting their potential health benefits and culinary uses. Thus, lycopene and β-carotene, two types of carotenoids, give tomatoes their high nutritious load along with Vit C, hinting to their antioxidant and anticancer properties.

The noteworthy nutritional and physicochemical analysis of the *Nendran* banana (*Musa* AAB) revealed a composition consistent with an unusual blend of energy value, protein, vitamins, minerals, and bioactive components than other fresh fruits. The moisture content contributes to its perishable nature, while the appreciable carbohydrate content, primarily composed of starch, makes it a good source of readily available energy, which is similar to findings on banana varieties known for their moisture retention properties by Kumar et al. 2019 (62.0%) and Kookal and Thimmaiah 2018 (66.23%) (28,161). Protein and fat content align with findings reported by Kumar et al. 2019 (0.83%) and Thatayaone et al. 2022 (128). Factors such as

genome type, variety, altitude, climatic conditions, and extra nutrients affect the protein content. Use of organic manures also affects protein content as they increase continuous nitrogen supply and synthesis of amino acids (162). The increased mineral buildup was indicated by the increased ash content insinuating the copious portrayal of inorganic matter comprising calcium, potassium, iron, phosphorus, etc., which are confirming the previous results of 1.54% and 1.61 mg/100g (28,161), although there was a higher value of 14.89% reported previously (128) which corresponds to the fact that triploids with plantain genome 'B' have a significantly higher ash amount than others. The TPC was found to be lower against a value of 0.82 mg GAE/g for nendran pulp but slightly higher when recorded against results by Thatayaone et al. 2022 (20 mg/100g) and Reshma et al. 2019 (0.156 mg GAE/g) for nendran banana (28,71,128). Due to variances in antioxidant profiles such as flavonoids and carotenoids, banana cultivars exhibit a sufficiently high degree of variability in their TPC, making them a favourable choice for the development of functional foods. These differences are ascribed to variations in genetic makeup and environmental influences, both of which have a major impact on the components' biochemistry (128). Vit C content is on par with previous reports, confirming the role as a good source of this vitamin, where a value of 12.57 mg/100g and a range of 28-35 mg/100g were noticed, in which the latter is higher compared to the obtained results, which could be due to the effect of nitrogenous fertilizers used as plants cultivated in environments with limited nitrogen supply possess significant quantities of Vit C (162). The high βC content corroborates the findings by Kumar et al. 2019 (12.6 μg/g) and Dhandapani et al. 2017 (13.6 μg/g), which attest that *Nendran* (AAB) has the greatest βC content amongst the major Indian banana cultivars (28,30). This high profile of βC is because of the presence of the "*Musa accuminata phytoene synthase* (*MaPsy*)" gene, and it's both isoforms, *MaPsy1* and *MaPsy2* (30). The higher TSS (compared to other banana cultivars) measured could be positively correlated to total carbohydrate as there exists a direct

relationship between TSS and carbohydrates content in bananas, which probably increases the palatability and higher consumer acceptance while making processed products (28,128). Further, the data are in agreement with the previous analysis results where TSS, acidity, and pH content ranging between 21.5-24%, 0.24-0.45%, and 4.50 were noticed, respectively (28,128,161,162). In general, the cultivar's genetic makeup determines the majority of its nutrient composition, with some environmental influences, altitude, maturity of the fruit, and additional nutrients (28,128). Thus, TPC, Vit C, and βC content hints at the potential antioxidant and anticancer properties of the cultivar selected.

The analysis of FOC revealed a promising nutritional profile with high protein content and a good balance of fat and carbohydrates, suggesting potential as a functional food ingredient. The high fat content makes it a rich source of omega-3 fatty acids (ALA), beneficial for heart health and inflammation reduction with anticancer properties. The low moisture content indicates excellent storage stability, while the ash content hints at the presence of valuable dietary minerals. The moisture content is affected by changes in the expeller process, the relative humidity and temperature at the mill, or the moisture content of the seeds at the time of the flaxseed pressing process. Results of proximate composition were in concord with values reported earlier by Ogunronbi et al. 2009, Mannucci et al. 2019 and Karnika et al. 2022 (31,163,164). The presence of high phenolic compounds (higher than SOC) underscores its potent antioxidant capacity, which is in agreement with earlier findings of 484.6 mg GAE/100g and 10.70 mg GAE/g TPC (31,164).

SOC's nutritional and physico-chemical examination demonstrates its potential as a useful agricultural byproduct rich in protein, fat, and dietary fiber (carbohydrates). The moisture content suggests good storage potential, while the ash content indicates the presence of essential minerals. Compared to previous results, the moisture, protein, fat, and ash content aligns with findings by Karnika et al. 2022, and Isarakul et al, 1993, who reported 37.16%

protein, 13.54% fat, and 8.72% ash content. The presence of high phenolic compounds suggests potential antioxidant properties, warranting further exploration towards the anticancer properties too. The TPC obtained was slightly lower than Karnika et al. 2002, who documented 5.83 mg GAE/g TPC. This could be because of variations in the oil extraction process in which certain chemical reactions that take place during oil extraction cause phenolic compounds that are insoluble to become soluble and result in the formation of many new compounds, including sesamol, vanillic acid, 3-hydroxy benzoic acid, filicinic acid, 4-hydroxy benzoic acid, and 3,4-dimethoxy phenol (31).

In summary, the current findings largely corroborate previous studies, reinforcing established nutritional profiles of these ingredients. However, slight variations in parameters highlight the influence of factors such as genetic buildup, cultivation conditions, processing, and measurement techniques.

6.2. Formulation of WMB formulations

The ingredients in the WMB formulations have been selected for their potential health benefits, particularly their antioxidant and anticancer properties. Whey's protein has been extensively studied for its anticancer properties (11). It is rich in amino acids and contains bioactive components such as lactoferrin and beta-lactoglobulin, which have been shown to possess anticancer activity. WP enhances GSH production, an antioxidant that protects cells from oxidative damage and supports immune function, potentially inhibiting cancer cell growth (165,166). WP possesses "antioxidant properties, the top protein quality rating qualities, and all of the EAAs," making it an excellent choice in this scenario (16,50,58). "EAA profile, protein efficiency ratio (PER), biological value (BV), simple digestion, assimilability, and solubility" are the reasons why WPs are touted as "wonder proteins." The optimum amino acid pattern exists in WPs, which are exceptionally high in "sulphur-containing amino acids

(methionine and cysteine) and branched-chain amino acids (leucine, isoleucine, and valine)" (15). These amino acids are crucial for "glutathione (GSH) biosynthesis as well as tissue growth and repair" (17). Lycopene from tomato has been linked to a reduced risk of various cancers, including prostate, lung, and stomach cancers. It works by neutralizing free radicals, thus preventing DNA damage, upregulating detoxification systems, inducing gap junctional communication, inhibiting cell cycle progression, arresting cell cycle in different phases, increasing induction of apoptosis, and inhibiting the proliferation of cancer cells (26,69,160,167). The multifunctional role of lycopene as a "nonsurgical aid in the treatment of oral diseases, including OSCC," is also well established (69). The Nendran banana fruit pulp is abundant in minerals like potassium, magnesium, and calcium, as well as Vit C, dopamine, phenols, flavonoids, and carotenoids (βC), along with antioxidant enzymes such as ascorbate peroxidase, catalase, peroxidase, and superoxide dismutase, all of which have significant implications in the prevention of cancer (28,29). Flaxseed and its oil cake are rich in TPC, ALA, and lignans, which are phytoestrogens with potential anticancer effects such as antioxidant, anticarcinogenic, antimutagenic, anti-proliferative, antiangiogenic, anti-invasive, antimigratory, and antiestrogenic properties, along with induction of cell death (32). Sesame seeds and its oil cake contain TPC and lignans such as sesamin and sesamol, which exhibit antioxidant and anti-inflammatory properties. These compounds can inhibit the growth of cancer cells by inducing apoptosis and cell cycle arrest. They also contain phytosterols, which have been shown to reduce the risk of certain cancers (168,169).

6.3 Quality analysis of WMB mixes

6.3.1 Proximate composition analysis of WMB mixes

A food product's compositional profile influences its textural qualities, shelf life, and physical and chemical characteristics in addition to determining its nutritional content. Hence, the

proximate analysis performed on the different treatments of WMB mixes on account of various parameters such as moisture, fat, protein, ash, carbohydrate, and energy determined are presented in Table 12. The moisture content of the WMB mixes is consistent with the previous findings on various whey-based products, which reported similar moisture levels in whey-based products, indicating that the variations in moisture content among the formulations are typical of different formulation strategies. The whey liquid's composition is influenced by many factors, including the milk source and the conditions under which it is processed (17,52). The results of test formulations except for protein and fat were following a similar trend with those reported for various types of fresh whey beverage formulations (Baccouche et al. 2013; Gad et al. 2013; Chatterjee et al. 2015 and Jakhar 2019). It was observed from the table 12 that the moisture content of the formulations decreased from treatment T4 to T1 as a result of a drop in the proportion of whey and an increase in tomato pulp extract. T1 had a moisture content slightly lower than control, while all other formulations had higher content, which was in proportion to increased whey content. This provides evidence that the WB T1 mix provides the same durability as the control sample, which is in agreement with previous findings on cookies made of whey and sucralose as well as biscuits fortified with WPC (172,173). These findings on moisture follow a similar trend with those reported for various types of fresh whey beverage formulations, although powdered WBs information was limited (106,111,113,171). Texture, taste, appearance, mouthfeel, microbial growth, and shelf-life are affected by the moisture content (172). The product must retain its desired properties up to the time of consumption. Therefore, ensuring optimal moisture content is a key aspect of quality control.

The fat content of the WMB mix test formulations ranged from 0.64±0.01 (T3) to 1.58±0.32% (T1), with a significant decrease observed as the whey percentage increased. The fat was found to be significantly lower in control (0.17±0.02%) than in T1, which may be attributed to the inclusion of whey, which improves the product's consistency and texture (172). These results

are comparable to those reported by Mykhalevych et al. 2024, who found that increasing WPI in formulations typically reduces the fat content due to the lower fat content of WPs compared to other ingredients (174). This trend reflects the typical outcome of substituting higher-fat ingredients with whey for various food formulations.

The protein content in the WMB mixes showed a notable trend that deviates from the general expectation that higher whey concentrations lead to higher protein levels. According to Table 12, the protein content ranges from 3.79±0.08 (T4) to 4.79±0.02% (T1), with T1 showing the highest protein content of 4.79%, despite having a lower whey concentration (45%) compared to other formulations. All the WB mixes had more protein content than control except T4. Thus, it was expected that the protein and fat content of the formulations would increase linearly as the amount of whey increased (171). Conversely, among the four test formulations, T1 with the least whey concentration exhibited the highest protein and fat content compared to other formulations, which may be due to the relatively equal proportion of whey and tomato pulp concentration than others, corroborating previous study results by Ovya 2020, which also reported a similar inference of high reference values for the formulation with equal blends of pomegranate juice and paneer whey (175). This unexpected result can thus be attributed to the composition and interactions of the ingredients, which could be due to the high proportion of tomato pulp in T1 (43%), which may have a synergistic effect with the WP, enhancing the overall protein content. Additionally, other factors, such as the protein content of the tomato pulp or specific interactions between the whey and tomato components, might contribute to the elevated protein levels. This may indicate that the large proportion of whey might not be effectively contributing to the protein content due to possible interactions or processing effects that affect protein stability and solubility. Various factors of processing parameters of heating and cooling rates, as well as pH, also significantly affect the WP aggregation. Following the standard heat treatment of whey (more than 72°C for 15–20 s), with the pH of the beverage

more than 4.6, the majority of WPs precipitate (13,176). And the pH of the study formulations was increasing from 4.60 to 5.13 for T1, followed by T2, T3, and T4, and this might have resulted in the slight decrease of protein content for the formulations with increased whey content.

Besides, the ash content ranged from 3.14±0.11 (T4) to 4.71±0.08% (C), in which higher whey concentrations often lead to lower ash content due to the lower mineral content of whey compared to other ingredients. Further, carbohydrate ranged from 85.70±0.39 (T1) to 87.80±0.17% (T4), and energy from 364.47±0.83 (C) to 376.18±1.40 kcal (T1), reflecting similar findings with the earlier results with whey alone and coupled with tomato (172,173). Pairwise comparison by DMRT showed that there is a statistically significant difference among the mean values of all test formulations with regard to proximate composition parameters. Beverage mixes showed a significant increase in protein, fat, and energy content greater than control, while ash values were significantly higher in control. These proximate profiles showed were corresponding to their respective ingredients' compositional analysis.

6.3.2 Nutritional composition analysis of WMB mixes

6.3.2.1 Bioactive component profile

From the table 12, it was found that the control formulation, with the highest concentration of tomato, exhibited the highest levels of Vit C, lycopene, βC, and TPC. This outcome aligns with existing literature that emphasizes the rich antioxidant profile of tomatoes, which are a well-known source of these bioactive compounds. Previous studies have consistently highlighted the role of tomatoes in providing a robust antioxidant capacity due to their high content of lycopene, βC, and phenolic compounds, which are crucial for scavenging free radicals and reducing oxidative stress (177,178).

Tomatoes and bananas, with their products, constitute a significant source of antioxidants, mainly Vit C, lycopene, and βC, along with various minerals, sugars, vitamins, and organic acids with therapeutic attributes. About 49 mg/100 g of Vit C was reported by Gould 1983, for concentrated tomato juice, which is approximately twice as much as in the experiment results for WP concentrate-enriched concentrated tomato juice (21.85 mg/100 g) represented by Rajoria et al. 2013. The current study findings of Vit C content was in agreement with the findings for a WB beverage enriched with tomato juice, which had 12.08 mg/100g of Vit C (118). Fruit maturity and variety, processing conditions, degree of oxidation, and the presence of other substances are the factors affecting the retention of Vit C (71,125). The combined effect of two sources (both tomato and nendran banana) rich in Vit C might be attributed to the high values in the test formulations.

In the control formulation, which has the highest tomato concentration of 88%, the lycopene content is also the highest at 4.42 mg/100g. This high lycopene level is directly attributable to the substantial tomato component, demonstrating a strong correlation between tomato presence and lycopene concentration. As the tomato concentration decreases in T1 to 43%, the lycopene content drops to 2.28 mg/100g. This significant reduction aligns with the expectation that less tomato means less lycopene. Further decreases in tomato concentration to 23% in T2 resulted in a lycopene content of 1.65 mg/100g, and a reduction to 13% tomato in T3 yields even lower lycopene levels at 0.58 mg/100g. This trend continues with T4, which has the lowest tomato concentration of 3% and the lowest lycopene content of 0.40 mg/100g. As the proportion of tomato in the formulations decreases, the lycopene content proportionally declines, underscoring the critical role of tomato in contributing lycopene to the WMB mixes.

Lycopene and βC, which are 11- and 9-conjugated carotenes, respectively, are the main pigments found in red tomato fruits (158). Lycopene-rich tomato products are claimed to be antioxidative and anticarcinogenic and can protect against a variety of malignancies in humans

(125). About 2.76-3.15 mg/100g of lycopene was reported for different treatments of the WB tomato beverage developed by Bangaraiah et al. 2014. However, lycopene concentrations in tomatoes can drop by 9–28% when they are thermally processed (177). Lycopene in tomatoes varies in composition as a result of varietal variances, climate-related conditions, agricultural factors, maturity stage, harvesting and post-harvest treatment, storage conditions, and transportation.

The highest βC content was accumulated in T1 at 0.29 mg/100g (Table 3), which supports the findings by other researchers on different WB beverages (93,106,179). Because boiled nendran banana pulp had used, the bioavailability of fat-soluble βC would also be increased upon consumption (30). The amount of plant material used for investigation as well as wet and dry weight basis analysis had significant impact on the overall phytochemicals quantified. Plant-derived polyphenols, offer profound value to the state of health.

Phenolic compounds are vital constituents of plants with redox properties that are responsible for antioxidant activity (180) which is mediated by plant hydroxyl groups. Numerous phenolic derivatives found in plants serve as natural antioxidants and are vital to the growth and reproduction of plants. They can be put up into several categories. The content of phenolic compounds in test formulations ranged from 0.14 mg GAE/g (T4) – 0.31 mg GAE/g (T1) (Figure 30). This results points out the richness of polyphenols in the formulation when compared with earlier results of different types of whey based beverages (85,181) and may be regarded as a prolific natural beverage mix possessing high phenolic compounds. This high phenolic content in the WMB mix may be attributed to the tomato pulp concentration and was proportional to the quantity added (182). Besides, the results of a pairwise comparison using DMRT revealed that there is a statistically significant difference between the mean values of all test formulations with regard to Vit C, lycopene, βC and TPC values at $p < 0.05$. Thus, the presence of these phytochemicals confirms the WMB to be of great medicinal value.

The data from Table 12 illustrates a clear correlation between tomato concentration and lycopene content in the WMB formulations. Lycopene, a carotenoid predominantly found in tomatoes and bananas, decreased as the tomato concentration reduces across the formulations with the banana content constant. In the control, which has the highest tomato concentration of 88%, the lycopene content is also the highest at 4.42 mg/100g. This high lycopene level is directly attributable to the substantial tomato component, demonstrating a strong correlation between tomato presence and lycopene concentration. This trend continues with T4, which has the lowest tomato concentration of 3% and the lowest lycopene content of 0.40 mg/100g. As the proportion of tomato in the formulations decreases, the lycopene content proportionally declines, underscoring the critical role of tomato in contributing lycopene to the WMB mixes. This decline is consistent with previous studies indicating that lycopene levels are closely linked to tomato content. For instance, a study by Rao and Agarwal 2000 highlighted that tomatoes are a primary source of lycopene and that reductions in tomato content directly lower lycopene concentrations (183). The significant p-value (**), reinforces the statistical reliability of these differences, confirming that the reduction in lycopene is significantly associated with the decreased tomato content.

6.3.2.2 Antioxidant assays

6.3.2.2.1 Total Antioxidant Capacity

The TAC values ranged from 156.53 μM Trolox/100g in T4 to 322.97 μM Trolox/100g in T1. This high antioxidant activity might be attributed to the to the levels of lycopene, β-carotene, and phenolic bioactive components combined from tomato pulp and nendran banana (184). Interestingly, the TAC in T1 surpassed that of the control, despite T1 having lower levels of Vit C, lycopene, βC, and TPC. This finding suggests that while tomato significantly contributes to the overall antioxidant capacity, whey also plays a crucial role, particularly in enhancing TAC. Whey is known for containing bioactive peptides, which have been demonstrated to

possess antioxidant properties. These peptides, formed during the hydrolysis of WPs, can neutralize free radicals and contribute to the overall antioxidant activity of food products (185,186). The higher TAC in T1 could be attributed to the synergistic effect between whey and tomato, where whey's antioxidant peptides complement the tomato's bioactive compounds, resulting in an enhanced antioxidant capacity. This synergistic interaction is crucial because it suggests that the combination of whey and tomato can offer superior antioxidant protection compared to using these ingredients separately. Such a combination could be particularly beneficial in the development of functional beverages aimed at promoting health and preventing chronic diseases linked to oxidative stress.

It is important to note that all ingredients other than whey are plant-based, and therefore contribute to higher values of lycopene, βC, and TPC content. Whey, being a dairy product, does not contain these compounds. The plant-based ingredients, particularly tomato, are rich sources of these antioxidants, and their presence in the control formulation significantly boosts these values. The absence of lycopene, β-carotene, and phenolic compounds in whey highlights the distinctive contributions of the plant-based components to the WMB mixes' antioxidant profile. However, whey's role should not be underestimated, as its bioactive peptides contribute significantly to the TAC, showcasing the importance of a balanced formulation that leverages both plant-based antioxidants and whey-derived bioactive compounds. This unexpected result highlights the role of WPs in enhancing TAC. Whey is known for its antioxidant properties, attributed to compounds such as lactoferrin and lactoperoxidase, which can scavenge free radicals and reduce oxidative stress (186). In comparison with previous studies, the results from this study reinforce the understanding that tomato's contribution to antioxidant capacity is primarily through its high levels of lycopene, βC, and phenolics. WPs have been shown to enhance the antioxidant properties of food products, not just through direct radical scavenging but also by modulating the body's endogenous antioxidant defence (11).

6.3.2.2.2 DPPH assay

The findings from the DPPH assay and IC_{50} values (Figure 32 and Table 13) underscore the significant role of a balanced combination of whey and tomato in enhancing the antioxidant properties of the WMB mixes. T1, which contains an optimal blend of whey and tomato, demonstrates superior antioxidant activity across all concentrations compared to other formulations. This synergy between whey and tomato ingredients is crucial, as WPs and peptides provide inherent antioxidant properties by scavenging free radicals and chelating metal ions (11,55,186). Tomato, rich in lycopene and βC, offers additional antioxidant benefits. However, when combined, these ingredients create a formulation that enhances the antioxidant capacity more effectively than when consumed separately. This synergistic effect is evident from the significantly higher DPPH inhibition and lower IC_{50} values in T1 compared to formulations with higher or lower concentrations of whey. The presence of whey enhances the antioxidant potential of tomato, and vice versa, resulting in a formulation that leverages the strengths of both ingredients to provide superior antioxidant benefits (187,188). This balance between whey and tomato ingredients in T1 demonstrates the importance of formulation in maximizing the health benefits of functional foods.

It is also important to note that all ingredients other than whey are plant-based, and therefore contribute to higher values of lycopene, βC, and TPC content. Whey, being a dairy product, does not contain these compounds. Instead, whey provides a different set of antioxidant compounds that complement the plant-based ingredients. This aligns with findings from previous studies that emphasize the significant antioxidant potential of whey and its bioactive components.

In summary, the results indicate that T1 has the highest antioxidant activity among the test formulations, followed by the control and T2, while T3 and T4 have significantly lower activities. These findings highlight the crucial role of whey in enhancing the antioxidant

properties of the WMB mixes and demonstrate the impact of ingredient composition on the overall antioxidant potential of the formulations. The balanced combination of whey and tomato in T1 significantly enhances the antioxidant properties of the WMB mixes compared to the individual consumption of whey and tomato.

6.3.2.2.3 Correlation of bioactive components with antioxidant activity

The Karl Pearson correlation coefficient between lycopene and TAC is 0.998 which indicate (0.998^2 = 0.996) 99.6 percentage positive relationship between lycopene and TAC and is significant at 1% level (Table 14). Similarly, the correlation coefficient between protein content and TAC is 0.997 which indicate (0.997^2 = 0.994) 99.4 percentage positive relationship between protein content and TAC and is significant at 1% level. Also, both Vit C and βC indicated 99.2 percentage positive relationship with TAC which is significant at 1% level. TPC had 98.8 percentage positive relationship with TAC at 1% significance.

Protein, primarily from whey, had weak negative correlations with Vit C, lycopene, βC, and TPC levels indicating that as the protein content increases, there is a corresponding decrease in Vit C, lycopene, βC, and TPC. However, the significant positive correlation between TAC and protein suggests that WP plays a pivotal role in boosting the antioxidant capacity, independent of the plant-based compounds. This dual contribution from both whey and plant-based ingredients underscores the importance of their combined use in formulating functional beverages with enhanced antioxidant properties.

6.3.2.2.4 Effect of bioactive components on TAC by multiple regression analysis

The multiple regression analysis is a powerful statistical tool for disentangling the complex relationships between bioactive components and TAC in WMB mixes. It provides a detailed understanding of how each component contributes to the overall antioxidant capacity, which is essential for optimizing the nutritional and functional qualities of the beverage mix. The data on Table 15 clearly demonstrates that based on standardized coefficient, protein content (0.761)

is the most important factor to extract TAC, followed by lycopene (0.614), βC (0.602), TPC (0.443) and Vit C (0.273) and thus each of the bioactive components plays a significant role in enhancing the TAC of WMB mixes. The particularly strong influence of protein, coupled with significant contributions from TPC and βC, underscores the importance of a balanced formulation that includes both whey and plant-based ingredients to maximize antioxidant properties.

WP, known for its high methionine, cysteine as well as leucine, isoleucine, and valine content, is a precursor to GSH, one of the body's most potent endogenous antioxidants (15,17). This mechanism likely contributes to the enhanced TAC observed in T1. They also have the ability to chelate metal ions (iron binding by lactoferrin) and scavenge free radicals directly, thereby augmenting the antioxidant potential of the formulation (17,61). On the other hand, tomato contributes through its rich content of lycopene, βC, Vit C, and TPC, all of which are powerful antioxidants that work by neutralizing free radicals and protecting cellular components from oxidative damage (26,69). The combined effect of whey and tomato in T1 is particularly significant. While whey provides a robust base of protein with inherent antioxidant properties, tomato adds a layer of protection with its high levels of carotenoids and phenolic compounds. This balanced combination results in a formulation that is more effective in combating oxidative stress than either whey or tomato alone. The regression analysis confirms this synergy, showing that while each component individually contributes to TAC, the collective impact is greater than the sum of its parts.

6.3.3 Physicochemical property analyses

The concept of water activity is used to determine the water that is used by the microorganisms for their growth and replication. This has been very useful in the preservation of foods. It is

defined as the degree of availability of free water in a food sample. Only this component takes an active part in the exchange with the ambient humidity and can possibly form the ideal medium for the microbial growth on the surface (189). The water activity of the formulations showed a clear increasing trend from T1 to T4, with T1 having the lowest value and T4 the highest. The values ranged from 0.25±0.01 in T1 to 0.41±0.01 in T4, indicating that higher whey concentrations resulted in higher water activity. The differences between all formulations are statistically significant ($p < 0.05$), as indicated by different superscript letters. This trend is consistent with findings in previous studies where higher protein content in formulations, such as whey, has been shown to bind more water, thereby lowering water activity. Low water activity is beneficial for shelf-life extension, as it reduces the likelihood of microbial growth (189).

T1 exhibited a significantly higher total solids content (96.13±0.09) compared to the control (95.65±0.11). The increase in total solids with added whey aligns with Solak and Akin 2014, who noted that whey powders contribute to the dry matter in food formulations (190). The differences are statistically significant ($p < 0.001$). From the data presented in Table 16, it can be observed that TSS, acidity, and viscosity decreased as the percentage of whey increased in the formulations and were proportional to the quantity of tomato pulp added. The pH values of the formulations increased from T1 (4.68±0.01) to T4 (5.13±0.01), with each formulation significantly different from the others ($p < 0.05$). This increase in pH correlates with the decreasing acidity observed in the titrable acidity values, which decreased from T1 (0.27±0.01%) to T4 (0.22±0.01%). The inverse relationship between pH and titratable acidity is well-established, as increased whey content buffers the acids present (191). Both parameters showed significant differences across all formulations ($p < 0.05$). Viscosity also showed a decreasing trend from T1 (10±0.01 cP) to T4 (7.00±1.15 cP), with significant differences between each formulation ($p < 0.05$). Various studies of WBs also reported similar observations

of physicochemical analysis of samples that varied based on the nature of ingredients added to whey beverages (111,175,192,193). The tomato juice enriched WB formulated by Rajoria et al. 2011 presented a higher acidity of 0.76%, whereas most of the other WBs depicted a similar physicochemical property analysis. The viscosity is a crucial parameter for judging the consistency of the prepared beverage samples. Viscosity decreased from the control (13.33±1.53) to T4 (7.00±1.15). Higher whey content leads to a less viscous mixture due to the lower viscosity of whey compared to fruit purees. This reduction in viscosity can improve the mouthfeel of the beverage (194). The differences are highly significant ($p < 0.001$). The decrease in viscosity with increasing whey concentration could be attributed to the dilution effect of whey on the overall mix. Colour being an important quality parameter for food formulations, especially beverages, control exhibited a high a* value, which indicates redness that is correlated with the higher tomato pulp and lycopene content followed by T1, T2, T3, and T4. The a* value decreased from the control (12.08±1.62) to T4 (4.22±0.01), indicating reduced redness with higher whey content, as the red lycopene pigment from tomato is diluted (177). L* value increased significantly from the control (34.05±1.53) to T4 (70.81±0.01), likely due to the pale colour of whey diluting the darker pigments of tomato and banana (195). The b* value also decreased from the control (31.27±1.25) to T4 (15.01±0.02), suggesting a reduction in yellowness which may be due to the dilution of yellow carotenoids from banana (196). Thus, fruit or vegetable juice addition can also significantly enhance the physicochemical characteristics of whey drinks.

6.3.4 Functional property analyses

The results in Table 17 highlighted the impact of ingredient composition on the functional properties of WMB mixes. Solubility increased significantly ($P<0.001$) from 65.5% in the control to 79.5% in T1. Whey's high protein solubility likely drove this, supported by its amphiphilic nature, which enhances water interaction, as noted by Hudson et al. 2000. The

differences in solubility can be attributed to the higher WP content in formulation T1 which possess superior solubility compared to the control due to the WP's ability to dissolve easily in water. WPs significantly enhance solubility and dispersibility due to their amphiphilic nature, which allows them to dissolve easily in aqueous solutions and disperse uniformly. According to Hudson et al. 2000, WPs improve solubility by forming molecular networks that enhance water binding and hydration during dissolution (197). In the case of the WSI, the higher whey content in T1 and T2 improves solubility due to the enhanced protein-water interactions from WPs. Wettability also showed a marked improvement from 21.5 seconds in the control to 10.25 seconds in T1. This suggests better hydration properties, likely due to whey's smaller molecular size, which allows faster interaction with water. The wetting time of the WMB mix was on par with values reported for protein rich instant rice beverage mix of 11.28 s (135). The sugar content was mainly responsible for this short wetting time. Tomato pulp's high fiber content in the control might have contributed to its slower wettability, similar to findings in Frusciante et al. 2007, who reported that tomato fiber can hinder rapid water uptake. Various factors including particle size, fat content, protein denaturation and resulting particle agglomeration affect the wettability. A higher level of heat denatured WP have a higher wettability, thus WP heat denaturation may be considered as a means to improve the wettability of milk proteins (198). Further, WPs with a non-agglomerated structure (undenatured) results in a higher retention of water.

In contrast, the control, which contains 88% tomato pulp, is likely influenced by the fibrous content of tomato, reducing solubility. The amphiphilic properties of WPs contribute to higher solubility and dispersibility by interacting efficiently with both hydrophilic and hydrophobic molecules. On the other hand, the fibrous nature of plant ingredients, especially tomato pulp, decreases solubility and increases wettability times due to their high cellulose and pectin content. The control formulation, dominated by plant-based ingredients like tomato and banana

pulp, displayed lower solubility and higher wettability, aligning with prior research indicating plant fibres' interference with hydration and dissolution properties.

6.3.5 Rheological properties

Viscosity is a key quality criterion for the preparation and utilization of various foods, especially beverages, and adds to ease of consumption. From this data, it was observed that with increasing shear rate (s^{-1}), all beverages' viscosity (η) generally decreased, thus following the non-Newtonian characteristics of pseudoplastic fluids. Besides, Table 18 shows the impact of temperature on the consistency coefficient (k) and flow behaviour index (n) of beverages. The k value for beverage ranged from 0.023 to 0.221. The Herschel-Bulkley model describes non-Newtonian fluids where shear-thinning occurs (pseudoplasticity). In Table 18, the low n values (e.g., T1: 0.415 at 25°C) signify strong pseudoplastic behaviour, while k values represent the consistency of the fluid. Higher whey content (T4) corresponds with higher k values (0.193 at 25°C), due to protein interactions contributing to thickening. As anticipated, the T1 formulation registered the highest viscosity, followed by T2, T3, and T4. The high viscosity of T1 could be due to its greater proportion of WPs and tomato pulp which was thus proportional to the quantity of solid components present. Further, the soluble fibres present in the formulations may absorb and hold onto moisture, thereby raising their viscosity (199). Further, at a narrow pH range near the protein isoelectric points, WPs and polysaccharides self-assemble into soluble complexes that exhibit unique functionality (146). Thus, viscosity decreased as the percentage of whey increased in the beverage formulations, corroborating previous study results by Arora 2022 for a whey based tomato soup.

The results showed that the consistency index dropped as the temperature rose which is in line with the findings for a protein-rich instant rice beverage mix developed by Swaminathan and Guha 2018. With $n<1$, the beverages displayed shear thinning behaviours for the Herschel-Bulkley model at the specified temperatures, and these observations were in line with the

results reported for synbiotic whey beverage by Kumar et al. 2021. This could be attributed to the molecule deterioration, deformation, subsequent molecular orientation, and interaction of the beverage's contents (202). The model of Herschel-Bulkley showed best fit ($r \geq 0.99$) for the beverages at a lower temperature of 10°C rather than 25°C.

6.3.6 Sensory evaluation

The sensory evaluation results for WMB formulations revealed a significant decline in sensory acceptability with increasing whey content (Table 19). Various senses of the product like visual appearance, smell, touch, taste and hearing are the way of knowing its acceptability through organoleptic evaluation. A few experienced judges simulate it as a way of the consumer response. The mean scores of WMBs varied in colour and appearance, flavour, mouthfeel, sweetness and overall acceptability differed significantly ($p < 0.05$). The formulations received scores in the range of 5.25-7.50, reflecting that the panellists were indifferent to some attributes of the products and liked moderately others. From these findings, T1 was preferred most for appearance and colour with the highest score which is in accord with the highest a* value with a positive correlation indicating more redness due to the tomato pulp concentration as well as taste, mouthfeel/after taste and overall acceptability. Besides, the observations also indicated that the panellists, on average, preferred the T2 formulation for the characteristic of consistency/texture (7.10±0.80) and shared the scores for flavour with the T1 formulation (6.60±1.35). A higher proportion of whey had a diminishing influence on sensory characteristics, which was similar to the findings of a whey-based tomato soup by Arora 2022. As the whey content increased (from T2 to T4), the sensory attributes, particularly appearance, taste, and overall acceptability, showed a downward trend. T4 (with 85% whey) had the lowest scores, particularly for appearance (5.45±1.19) and taste (5.25±1.07) likely due to a chalky texture and WP-associated off-flavours, consistent with Komeroski and Oliveira 2023, which found that high whey content could result in undesirable sensory attributes in the finished

products (203). Moreover, previous experiments have shown that tomato juice beverages enriched with whey were highly acceptable to the sensory team (118,125). Further, the post hoc analysis by DMRT demonstrated that there is a statistically significant difference among the mean values of test formulations with regard to all sensory scores' parameters except flavour and mouthfeel/after taste. These results were almost in agreement with previous literature results for various whey based beverages by Baljeet et al. 2013; Chatterjee et al. 2015; Punnagaiarasi et al. 2017; and Karthikeyan 2018 in which the addition of fruits or vegetables to whey drinks increased the overall sensory attributes and consumer acceptability and thus paved the way for better utilisation of whey. Industrial applications of whey for the creation of whey-tomato based beverages may find this information to be crucial.

6.3.7 Mineral profile of T1 WMB mix

The mineral content of the T1 WMB mix reflects the contribution of whey, especially in calcium and phosphorus. Whey is also known for enhancing mineral absorption due to the presence of lactoferrin, a protein that binds to minerals like iron and enhances their bioavailability. In comparison with other popular food products, the T1 WMB mix shows moderate levels of calcium and phosphorus compared to dairy-rich products. However, it exhibits higher potassium levels, which make it particularly beneficial for cardiovascular health. A study by Berrazaga et al. (2019) highlighted the importance of WPs in enhancing mineral bioavailability, particularly calcium and phosphorus. The high potassium content of the T1 WMB mix also mirrors findings from a study on potassium-rich diets, which emphasized their role in reducing hypertension and promoting heart health.

Trace minerals, though required in small amounts, play vital roles in various physiological and biochemical functions, particularly in the modulation of antioxidant and anticancer activities. The T1 WMB mix contains 22.3 mg of zinc, contributing to its antioxidant properties. Zinc acts as a cofactor for superoxide dismutase (SOD), an enzyme that neutralizes reactive oxygen

species (ROS) and protects cells from oxidative damage. The minerals Zn, Cu and Mn serve as cofactors for various antioxidant enzymes, notably superoxide dismutase (SOD) in its different forms (CuZn-SOD and Mn-SOD), which are critical in defending the body against oxidative stress.

6.3.8 Protein quality analyses of T1 WMB mix

Some popular techniques for figuring out the protein quality of food samples are the information pertaining to their amino acid content, and *in vitro* protein digestibility values. These analyses were carried out for T1 WMB mic and results are depicted below.

6.3.8.1 Amino acid analysis of T1 WMB mix

The amino acid profile of the T1 WMB mix, as presented in Table 20, provides crucial insight into the nutritional quality of the formulation. Whey, known for its rich protein content and balanced amino acid profile, plays a significant role in influencing the amino acid composition of the WMB mix.

Glutamic Acid and Aspartic Acid are found in moderate quantities (0.1919 mg/g and 0.1936 mg/g respectively), both being non-essential amino acids known for their roles in neurotransmission and energy metabolism. They also act as precursors for other amino acids and contribute to protein structure and functionality. McIntosh et al. 1995 found that WP inhibits the growth of cancer cells by modulating immune responses and enhancing the body's ability to repair DNA damage. This is attributed to the high content of amino acids like cystine and glutamic acid, which promote glutathione production (204,205). Histidine (0.4649 mg/g) is an essential amino acid with important roles in the synthesis of haemoglobin and histamine. Its presence in significant quantities enhances the beverage's value for immune and anti-inflammatory responses. Tyrosine (0.8286 mg/g), one of the highest-concentration amino acids in the mix, is a non-essential amino acid that serves as a precursor for neurotransmitters such as dopamine, epinephrine, and norepinephrine.

Leucine (0.4888 mg/g) and Valine (0.3603 mg/g), both branched-chain amino acids (BCAAs), are crucial for muscle repair, protein synthesis, and energy production during exercise. Whey is particularly rich in leucine, which is known to stimulate mTOR (mechanistic target of rapamycin) pathways, thus promoting muscle protein synthesis (206). An extra advantage of leucine for cancer cachexia therapy is its capacity to induce MPS at lower doses.

Methionine (0.0091 mg/g) and Cystine (0.0743 mg/g) are sulphur-containing amino acids. Methionine is essential for methylation processes, while cystine is involved in antioxidant defence through the synthesis of glutathione. The presence of these amino acids, although in low amounts, suggests the potential antioxidant capacity of the WMB mix. Bounous et al. and their several studies demonstrated that WP supplementation leads to increased glutathione levels, enhancing the body's defence against oxidative stress and cancer development (166,207–210).

Lysine (0.0232 mg/g), though present in small quantities, is an essential amino acid important for calcium absorption, immune function, and collagen formation. Lysine is often the limiting amino acid in plant-based proteins, but whey's presence ensures its sufficiency in the T1 mix.

6.3.8.2 *In Vitro* Protein Digestibility assay

The IVPD metric is crucial for figuring out how nutrient-dense and adequate certain foods are. WPs are known for their high digestibility due to their solubility and rapid absorption, making them one of the best proteins for bioavailability. When compared to other dietary proteins, WP possesses "antioxidant properties, the top protein quality rating qualities, and all of the EAAs," making it an excellent choice in this scenario (16,50,58). As quoted above, WPs were renowned as "wonder proteins" (16). "EAA profile, protein efficiency ratio (PER), biological value (BV), simple digestion, assimilability, and solubility" are the reasons why WPs are touted as "wonder proteins." Besides, Renner (1992) documented that "WPs have greater BV (104) compared to casein (77) and whole eggs (100). They also have better PER and net protein utilization (NPU)

than casein." "While the NPU is 92 for WPs, 76 for casein, and 94 for whole eggs, the PER of WPs is 3.6 compared to 2.9 for casein and 3.8 for whole eggs (15)" (Table 2). Besides, since whey contains the "highest concentration of readily absorbed and digestible EAAs," it can be integrated into body cells more quickly than other dietary protein sources and improves plasma amino acid and protein synthesis (59).

6.3.9 Microbial evaluation

The microbial evaluation studies have shown that T1 WMB mix was shelf stable for upto 3 months with regard to total bacterial count, total coliform count, salmonella and yeast and mold count and the observed values were within the limits of the recommendation of FSSAI standards of quality for whey-based powders.

This microbial evaluation suggests that while refrigeration and proper packaging can significantly reduce microbial proliferation in T1 WMB mix, certain packaging types (especially metallic pouches) provide better protection over time. The absence of harmful pathogens such as coliforms, Salmonella, and fungi reflects good manufacturing practices. However, for prolonged storage, attention should be given to improving the microbial stability of the product, particularly under ambient conditions. These findings are crucial for recommending appropriate storage conditions and packaging solutions for whey-based beverages to ensure safety and quality during shelf life.

The presence of viable bacterial colonies on NA suggests that general microbial growth occurred over time, particularly under ambient conditions. After 90 days, the highest microbial load was observed in the glass bottle (108×10^3 CFU/g), followed by the plastic pouch (67×10^3 CFU/g) and the metallic pouch (58×10^3 CFU/g). This trend could be attributed to the differential permeability and interaction of these materials with the environment. Interestingly, the refrigerated samples showed significantly lower counts, indicating that low temperatures

slow down microbial growth, with the metallic pouch exhibiting the lowest viable count (34 × 10^3 CFU/g) at 90 days.

The absence of significant microbial growth on EMB and SS plates indicates the absence of coliform bacteria and Salmonella/Shigella contamination, which are indicators of sanitation and hygiene. These results suggest that the formulation and packaging process were relatively clean and free from contamination by harmful pathogens. The low fungal counts (all < 50 CFU/g) suggest minimal or no fungal contamination, highlighting the microbiological stability of the product in terms of mold and yeast growth over time, even after 90 days.

Glass bottles showed the highest microbial growth in both ambient and refrigerated conditions, which could be attributed to possible exposure to light or a less effective sealing mechanism compared to pouches. Plastic pouches and metallic pouches offered better protection, especially at refrigerated temperatures, with metallic pouches showing the best performance in limiting microbial growth (lowest counts at 90 days). Microbial growth was much higher at ambient temperatures compared to refrigerated storage, reaffirming that refrigeration is an effective method to extend the shelf life of whey-based beverages.

6.3.10 Shelf-life studies

Based on the microbial evaluation studies, the shelf-life studies of T1 WBM was conducted by storing T1 in metallic pouch for 90 days' time period at refrigerated condition (4±1°C) and it was shown that as the time increases, various physicochemical parameters including TSS, acidity was found to be increasing while pH and sensory attributes were found to be decreasing. The table 21 shows the effect of storage on physicochemical parameters of the T1 WMB mix over 90 days, including Total Soluble Solids (TSS), acidity, and pH. These parameters are crucial indicators of the quality, stability, and potential shelf life of food products. The data presented here highlights changes observed at 0-, 45-, and 90-days during storage.

The results from the physicochemical analysis of the T1 WMB mix stored over 90 days reveal significant changes in TSS, acidity, and pH. The increase in TSS suggests concentration due to moisture loss or solute redistribution, which may enhance the sweetness and overall intensity of the beverage's flavour. However, this concentration effect can also alter the texture and mouthfeel, potentially making it less appealing if the changes are too drastic.

The increase in acidity and the corresponding decrease in pH indicate that the T1 WMB mix is undergoing biochemical changes during storage, likely due to enzymatic activities or microbial action. A rise in acidity may be beneficial in preventing microbial spoilage, as higher acidity is less conducive to the growth of spoilage organisms. This aspect could be emphasized when recommending storage conditions, as refrigeration or further adjustments in acidity could prolong shelf life.

However, the decrease in pH could lead to flavour changes, with the product becoming more sour or tangy over time, which may not be favourable for all consumers. The balance between maintaining microbial stability and preserving desired sensory characteristics should be considered in future formulations or processing adjustments.

Several studies have indicated that food products, especially those containing proteins, undergo sensory changes during storage. Whey protein-based products are known to suffer from oxidation of lipids and Maillard reactions, which degrade both flavor and appearance (Rufián-Henares & Morales, 2007). In a study on shelf stability of protein-fortified beverages, there was a significant reduction in flavor scores due to protein denaturation and chemical changes during storage (Drake et al., 2016). Similar trends were observed in the T1 WMB mix, where sensory parameters, especially appearance, and taste, declined over time.

6.3.10.1 Shelf life of T1 WMB mix

Given the data from sensory and physicochemical analyses, the shelf life of the T1 WMB mix can be considered to be around 90 days, as the product remains organoleptically acceptable and

physiochemically stable up to this point. Beyond 90 days, further declines in sensory quality (especially in taste and appearance) and potential increases in acidity may render the product less desirable for consumers. Therefore, 90 days can be recommended as the upper limit for the shelf life of the T1 WMB mix under the given storage conditions.

6.3.11 Anticancer activity assays

6.3.11.1 *In vitro* cytotoxicity of ingredients against KB OC cells

The results indicated a dose-dependent cytotoxicity of WP against KB OC cells, with significant reductions in cell viability at higher concentrations. Several studies corroborate these findings, emphasizing WP's anticancer potential through its bioactive components (11). The cytotoxic effect of WP is primarily driven by its bioactive components, as WPs, are known for its high methionine, cysteine as well as leucine, isoleucine, and valine content, and is a precursor to GSH, one of the body's most potent endogenous antioxidants (15,17). GSH plays a key role in detoxification and oxidative stress regulation. The high concentration of cysteine and other peptides can trigger oxidative stress and apoptosis in cancer cells, leading to cell death. A study by Kent et al. 2003 showed that WP effectively inhibited the proliferation of prostate cancer cells by inducing apoptotic cell death that stems from its ability to induce apoptosis via the caspase pathway and reduce oxidative damage (165). Another study by Parodi 2007 confirmed that bioactive peptides in whey exhibit anticancer effects by modulating the redox environment within the cells (211).

They also have the ability to chelate metal ions (iron binding by lactoferrin) and scavenge free radicals directly, thereby augmenting the antioxidant potential (17,61). Additionally, bioactive peptides in whey modulate cell signalling pathways, enhance immune responses, and suppress cancer cell proliferation through mitochondrial dysfunction, all contributing to reduced cell viability at higher concentrations (212). Studies have confirmed that WP's ability to increase

glutathione levels also makes cancer cells more susceptible to apoptosis compared to healthy cells.

Further, the results indicated a dose-dependent cytotoxicity of tomato against KB OC cells, with significant reductions in cell viability at higher concentrations. This dose-dependent reduction in cell viability is a crucial finding that aligns with previously reported studies on the anticancer potential of tomato and its bioactive compounds. Previous studies have consistently highlighted the role of tomatoes in providing a robust antioxidant capacity due to their high content of lycopene, βC, Vit C, and phenolic compounds, which are crucial for scavenging free radicals and reducing oxidative stress (177,178). Lycopene and βC, which are 11- and 9-conjugated carotenes, respectively, are the main pigments found in red tomato fruits (158). Lycopene-rich tomato products are claimed to be antioxidative and anticarcinogenic and can protect against a variety of malignancies in humans (125).

Gupta et al. 2015 pointed out the multifunctional role of lycopene as a “nonsurgical aid in the treatment of oral diseases, including OSCC.” Lycopene has been linked to a reduced risk of various cancers, including prostate, lung, and stomach cancers. It works by neutralizing free radicals, thus preventing DNA damage, upregulating detoxification systems, inducing gap junctional communication, inhibiting cell cycle progression, arresting cell cycle in different phases, increasing induction of apoptosis, and inhibiting the proliferation of cancer cells (26,69,160,167) (Fig. 7). It was also found to raise the amounts of the p53 protein in cancer cells, arrest the cell cycle via modulating cell cycle regulatory proteins, and alter mitochondrial function. More than that, the Ras-dependent activation of NF-κB was reduced by lycopene, which was accompanied by an inhibition of ROS production and a decrease in the phosphorylation of JNK, ERK1/2, and p38. And lycopene suppressed Akt activation and regulated downstream targeted molecules such as cyclin D1 and p27 to inhibit cell proliferation (26). According to a comprehensive study by Singh and Goyal 2008, lycopene in tomatoes has

been shown to be protective against a number of malignancies in humans, including colorectal, prostate, breast, lung, and pancreatic cancers (70).

Bananas are packed with functional, health-promoting nutrients such as Vit C, provitamin A carotenoids, potassium, and fibre (27). The Nendran fruit pulp is abundant in minerals like potassium, magnesium, and calcium, as well as Vit C, dopamine, phenols, flavonoids, and carotenoids (βC) along with antioxidant enzymes such as ascorbate peroxidase, catalase, peroxidase, and superoxide dismutase, all of which have significant implications in the prevention of cancer (28,29). By scavenging free radicals, βC has been demonstrated to have protective benefits against oxidative stress and functions as a precursor to vitamin A (213). The DNA damage that causes cancer may be prevented by this antioxidant activity. Given that free radicals and oxidative stress play a major role in the formation of cancer, the lethal effects of βC in the KB OC cells are further supported by its activity. Further, Vit C is also a potent antioxidant, neutralizing free radicals and preventing oxidative damage to DNA. Several studies have demonstrated that Vit C can inhibit the proliferation of cancer cells and induce apoptosis through ROS generation (214).

The concentration-dependent reduction in cell viability underscores the anticancer potential of FOC, particularly in concentrations of 500 µg/ml and 1000 µg/ml. It also boasts a considerable total phenolic content (TPC), ranging from 776 to 2255 mg per 100 grams, with variations based on the extraction solvent used. Additionally, FOC is also rich in PUFAs, particularly ALA (omega-3) and conjugated linolenic acid, offering a myriad of health benefits, including maintaining low levels of LDL, along with exhibiting anticancer, antihypertensive, antidepression, antiaging, and antiarthritis effects (31). Besides, lignans found in FOC have been associated with a reduced risk of certain cancers, including breast, prostate, and colon cancer, due to their antioxidant, anticarcinogenic, antimutagenic, anti-proliferative, antiangiogenic, anti-invasive, antimigratory, and antiestrogenic properties, along with

induction of cell death (32). In addition, there are several indications showing that eating foods high in dietary fiber lowers the risk of colon cancer. Increased intake of foods and dietary supplements high in omega-3 fatty acids may also reduce the risk of colorectal and breast cancers, according to some research (75).

6.3.11.2 *In vitro* cytotoxicity of T1 WMB mix against KB OC cells

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 (215). In this specific study, the cytotoxicity of developed T1 WMB mix was evaluated using the MTT assay, and it was found that the sample showed anticancer activity against KB OC cells, wherein, fortunately, it failed to exhibit any cytotoxic impact on 3T3-L1 cells (Figs. 41 and 43). 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 oxidoreductase enzyme (216). A solubilization solution like DMSO can be used 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 T1 concentration, and 200 μg mL^{-1} of T1 formulation was required to reduce KB OC cells' vitality to 50% (IC_{50}) of the starting population (Fig. 41). Following a 24-hour treatment period, T1 formulation was found to exhibit dose-dependent cytotoxicity against KB OC cells but was found to be safer without any effect on 3T3-L1 normal cells with various concentrations tested, including 10, 20, 40, 80, 160, and 320 μg mL^{-1} for both types of cells (Figs. 41 and 43). 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 (217). Another study on the antiproliferative and apoptotic effects of probiotic whey dairy beverages in human prostate cancer cell lines (PC-3 and DU-145) also showed cytotoxic activities against both cell lines (218). These data gathered thus point to the deployment of whey-based drinks to prevent cancerous cell proliferation.

6.3.11.3 Cell cycle analysis

Reproduction of cells requires cell division, with the production of two daughter cells. The most obvious cellular structure that requires duplication and division into daughter cells is the cell nucleus - the repository of the cell's genetic material, DNA. With few exceptions, each cell in an organism contains the same amount of DNA and the same complement of chromosomes. Thus, cells must duplicate their allotment of DNA prior to division so that each daughter will receive the same DNA content as the parent. The cycle of increase in components (growth) and division, followed by growth and division of these daughter cells, etc., is called the cell cycle. The two most obvious features of the cell cycle are the synthesis and duplication of nuclear DNA before division and the process of cellular division itself -mitosis. These two components of the cell cycle are usually indicated in shorthand as the “S phase” and “mitosis” or “M”. When the S phase and M phase of the cell cycle were originally described, it was observed that there was a temporal delay or gap between mitosis and the onset of DNA synthesis, and another gap between the completion of DNA synthesis and the onset of mitosis. These gaps were termed G1 and G2, respectively. The cycle of G1 → S → G2 → M → G1, etc., is shown schematically in Figure 47.

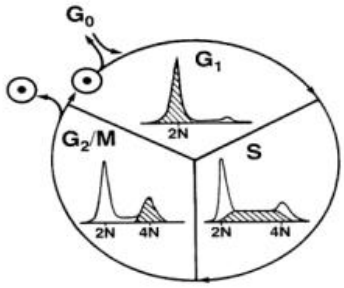
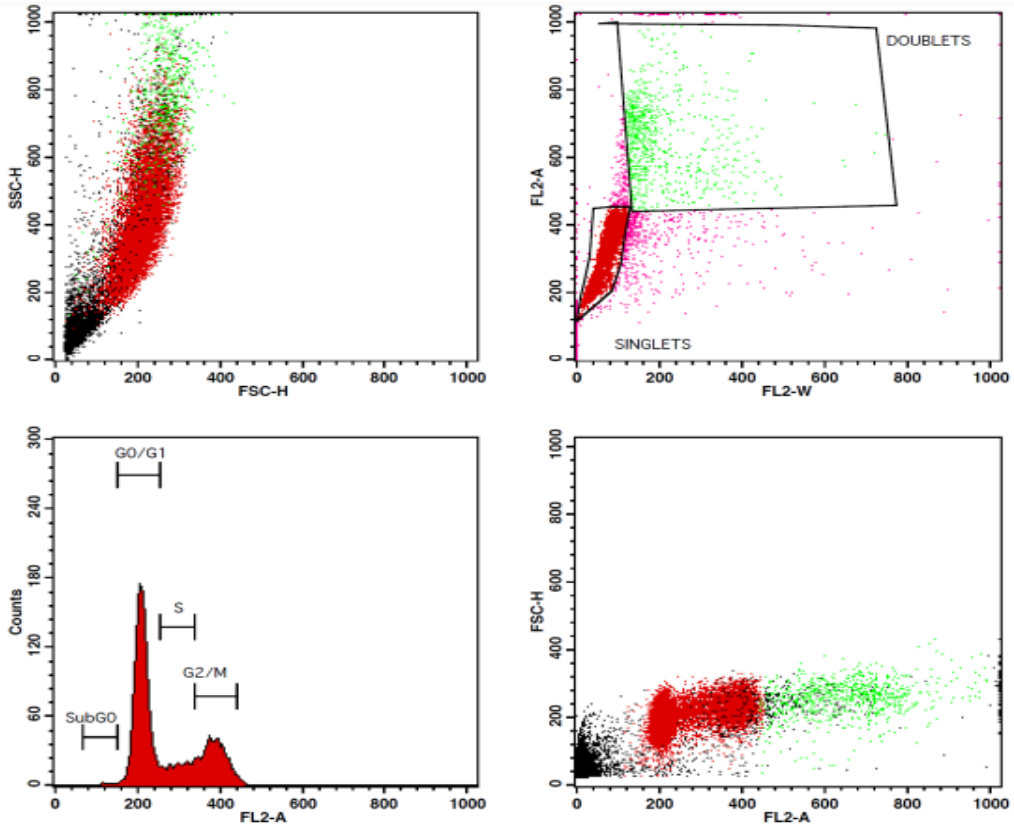


Figure 47. A schematic diagram of the cell cycle, showing flow cytometric components of each phase.

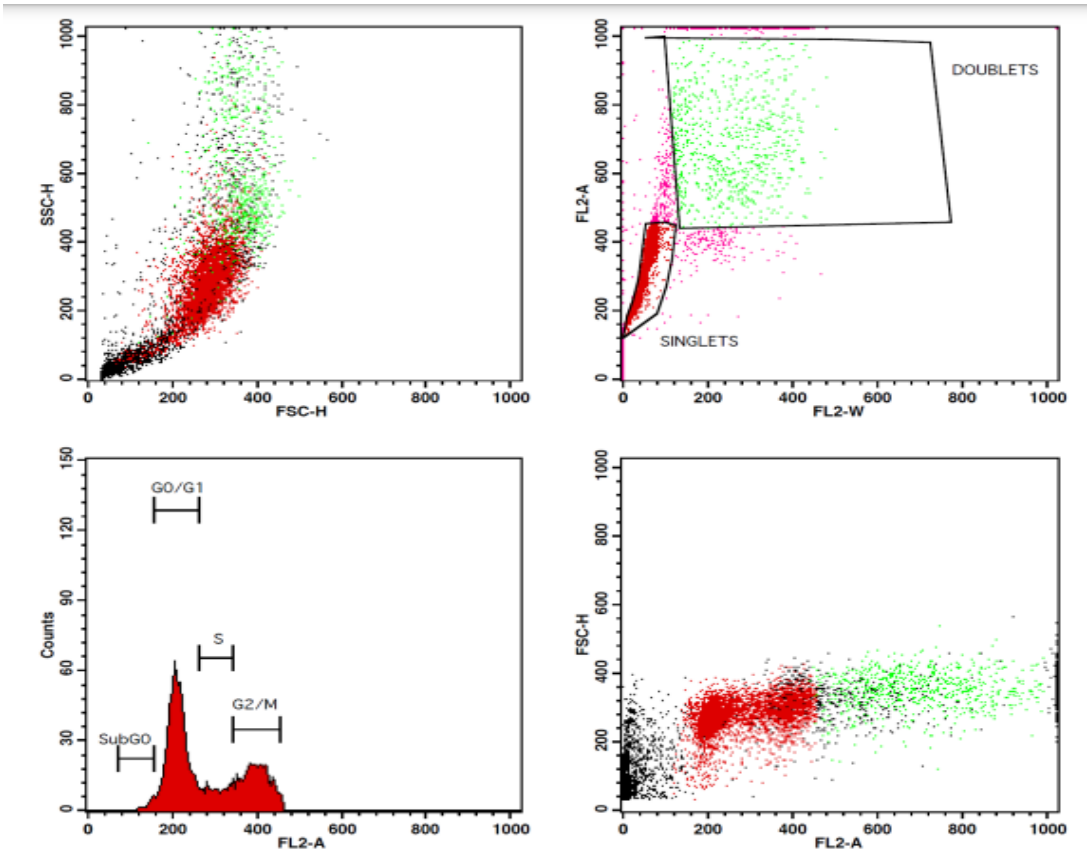
One of the earliest applications of flow cytometry was the measurement of DNA content in cells. This analysis is based on the ability to stain cellular DNA in a stoichiometric manner. A variety of dyes are available to serve this function, all of which have high binding affinities for DNA. The location to which these dyes bind on the DNA molecule varies with the type of dye used. The most common DNA-binding dye in use today is the blue excited dye Propidium Iodide (PI). PI is an intercalating dye that binds to DNA and double-stranded RNA (and is thus almost always used in conjunction with RNaseA to remove RNA). When diploid cells which have been stained with a dye that stoichiometrically binds to DNA are analyzed by flow cytometry, a “narrow” distribution of fluorescent intensities is obtained.



Histogram Statistics

Marker	Left, Right	% Gated	% Total	Mean	CV
All	0, 1023	100.00	65.92	267.89	29.82
SubG0	68, 154	0.31	0.20	134.16	10.21
G0/G1	152, 255	60.57	39.93	209.81	7.60
S	255, 340	13.07	8.62	299.90	8.34
G2/M	340, 442	25.93	17.09	385.98	6.40

Figure 48: Flow cytometry plots of KB OC cells treated with T1



Histogram Statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	62.73	278.53	31.41
SubG0	72, 158	69	1.38	0.87	146.94	7.66
G0/G1	156, 264	2794	55.88	35.05	210.93	10.16
S	264, 344	652	13.04	8.18	305.21	7.85
G2/M	344, 456	1512	30.24	18.97	396.91	7.06

Figure 49: Flow cytometry plots of KB OC cells treated with standard Doxorubicin

Cell division is a highly regulated process that is responsible for the appropriate division of a cell into two daughter cells. The cell cycle combines DNA replication with chromosomal segregation in an oscillatory manner. Cell cycle arrest was the first identified effect of HDAC inhibitors on cancer cells. HDAC inhibitors are capable of causing cell cycle arrest in a broad range of cells, including numerous forms of cancer and both cancerous and noncancerous cells. Another study by Murali et al., stated that camel WPH induced G2/M cell cycle arrest in human colorectal carcinoma (30). A study by Tutku et al has also reported that whey protein derivatives induced G2/M phase arrest in the MCF-7 cells (31). As there are very limited

studies that determine the cell cycle arrest by whey formulations, this study stands novel and is the first study to determine the cell cycle arrest against squamous cell carcinoma.

Cell cycle arrest or regulation is a critical approach to an increased likelihood of anticancer therapeutics. It is a regulatory mechanism in living cells to pause or slow down cell division for repair, differentiation, or other reasons. In the current investigation, the developed WMB T1 was able to stop the cycle in the KB OC cell line at the G0/G1 phase 24 hours after treatment. While T1-treated cells had 60.57%, 13.07%, and 25.93% of their cells at G0/G1, S, and G2 phases, respectively, it became apparent that 91.92% of the untreated cells were present in G0/G1, 4.68% in the S phase, and 2.75% in the G2 phase (Fig. 45&48). The percentage of cells in the G0/G1 phase had increased significantly ($p < 0.05$) in comparison to the control (untreated cells). Moreover, T1's efficacy was on par with that of doxorubicin, which served as the primary positive control in the trial and was referred to as the standard. (Fig. 45&49) To summarize, T1 has shown S phase arrest of 13.07% and G2 phase arrest of 25.93% in KB OC cells, which is almost equivalent to doxorubicin cell cycle arrest in S phase and comparable to G2 phase arrest. A number of modes of action are implicated in the various patterns of cell cycle arrest that have been seen as a pharmacological endpoint, indicating the involvement of chemicals in each extract in mediating this activity (219). Our findings provide credence to the theory that the extracts prevent cancer cells from proliferating by preventing the advancement of the cell cycle, suggesting that the extract might have therapeutic potential. The outcomes of the study corroborate the theory that the combined action of phytochemicals and WPs may have caused oxidative stress, which ultimately resulted in the induction of KB OC cell death (45, 46). Cell cycle arrest investigation in whey-based beverages or beverage mixes and in KB OC cells might be an unconventional application, and hence specific research on this topic is less studied.

6.3.11.4 Cell death analysis

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages. Once triggered, apoptosis proceeds with different kinetics depending on cell types and culminates with cell disruption and the formation of apoptotic bodies. A critical stage of apoptosis involves the acquisition of surface changes by dying cells that eventually results in the recognition and uptake of these cells by phagocytes. Different changes on the surface of apoptotic cells such as the expression of thrombospondin binding sites, loss of sialic acid residues, and exposure of a phospholipid like phosphatidylserine (PS) were previously described (25).

Phospholipids are asymmetrically distributed between the inner and outer leaflets of the plasma membrane with phosphatidylcholine and sphingomyelin exposed on the external leaflet of the lipid bilayer, and phosphatidylserine predominantly observed on the inner surface facing the cytosol. Exposure of PS on the external surface of the cell membrane has been reported for activated platelets and senescent erythrocytes. Recently, it was shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose PS which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark (early and widespread) in detecting dying cells. Annexin V, belonging to a recently discovered family of proteins, the annexins, with anticoagulant properties has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca2+ and shows minimal binding to phosphatidylcholine and sphingomyelin (26).

Changes in PS asymmetry, which is analyzed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis occurred and before membrane integrity has been lost. By conjugating FITC to Annexin V it is possible

to identify and quantitate apoptotic cells on a single cell basis by flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence) and the propidium iodide (red fluorescence) allows the discrimination of intact cells, early apoptotic and late apoptotic or necrotic cells (27). In the study, the apoptotic potential of whey-based formulation against the KB OC cell line which is isolated from the tongue of a 25-year-old male patient with squamous cell carcinoma was performed. WP is proven to have benefits for cancer patients. It is also demonstrated that protein hydrolysis of whey may improve anticancer efficacy. Hence, the test formulation made of whey (T1) in the present study was evaluated for its anticancer potential against KB OC cells.

Biochemically, cancer is signified by a range of hallmarks consisting of the dissolution of intracellular substrates, DNA distortion, apoptosis, phosphatidylserine (PS) externalization, and subsequent immune evasion. In cancer cells, PS can be exposed on the outer leaflet, which acts as a signal for phagocytosis, while it is primarily found on the inner leaflet of the plasma membrane in healthy cells (47, 48). When PS is conjugated fluorescently, flow cytometry could be used to detect asymmetrical loss in PS when it is labelled with Annexin V, which is reported to attach solely to PS (223). In a similar manner, the WMB mix T1 was used to treat KB OC cells, and the phases of apoptosis were determined by double labelling with Annexin V and PI (Fig. 45). A total of 64.07% of the cells underwent apoptosis after being treated with T1 formulation for 24 hours; on the other hand, doxorubicin drove 49.18% of the cells to undergo apoptosis (Fig. 46). Further, T1 induced 48.44% early apoptosis and 15.63% late apoptosis, while doxorubicin induced 22.38% early apoptosis and 26.80% late apoptosis. The study's results are consistent with the idea that phytochemicals and WPs combined effect might induce reactive oxygen species and oxidative stress, which led to the KB OC cell line's demise (45, 46). The whey protein anticancer effect was evaluated against melanoma B16F10 cells as a model and noted that caspase-3 expression was increased significantly in the whey protein

isolate-containing media (28). The effect of whey protein against PC12 rat pheochromocytoma cells was also studied at a dose of 100 to 400 µg hydrolysate/ml and found significant results (29). As the studies reported the potential of whey, the present study test formulation tested against squamous cell carcinoma cells proved once again the effectiveness of whey which is a good observation. As there is a less common implementation of the Annexin V-FITC kit and associated cell death analyses in whey-based beverages or beverage mixes and in KB OC cells, specific studies on this topic are also limited.

6.3.11.5 Cell morphology analysis

The study of cell morphology is vital to know cell behaviour by tracking the intracellular molecules and organelles and also the electrochemical deformation of cells. In the present study, Acridine orange Ethidium bromide dual stain method is used to detect the deformation of cells. Acridine orange is an important dye that stains both live and dead cells. Ethidium bromide stains only cells that have lost their membrane integrity. Hence live cells appear green in colour. Early apoptotic cells will take green colour and exhibit bright green dots in the nuclei whereas late apoptotic cells will take ethidium bromide and stain orange and show condensed and fragmented nuclei. Necrotic cells stain orange and have similar nuclear morphology to viable cells with no chromatin condensation. This dual acridine orange/ethidium bromide fluorescent staining can be used to identify apoptosis-associated changes in cell membranes during the process of apoptosis (32,33). In the present study, this method is used to detect apoptosis in KB oral cancer cells. As per the study observations, it was noted that T1 test formulation at 200 µg/ml induced early apoptosis in the KB cancer cell line, and doxorubicin at 25 µM showed induction of late apoptosis of KB oral cancer cells. To our knowledge, this is the first study to report that whey formulation can induce apoptosis in KB OC cells.

To verify the findings from the cytotoxic research, the morphological alterations in the KB OC cells were assessed using AO-EtBr staining, as it has been hypothesized to be one of the best

economic and convenient choices for distinguishing between apoptotic and necrotic cells (224). In this particular observation, early apoptotic cells were showcased to have green-yellow fluorescence, which is indicative of a compacted or shattered nucleus containing chromatin Figure 45. On the other hand, live cells had a characteristic green nucleus, necrotic cells illustrated a distinct red nucleus, whereas cells in the final stages of cell death featured yellow-orange fluorescence, signifying chromatin breakage and necrosis of the cell. The outcomes imply that treatment of KB OC cells with T1 formulation transformed the cell structure, featuring green-yellow fluorescence, as seen in Figure 45, which points out the stages of early apoptosis, whereas the shift in colour from green-yellow to yellow-orange fluorescence in doxorubicin-treated cells illustrated late-programmed cell death cells, and the control cells (untreated) seemed to have the characteristic green fluorescence, reflecting the normal live cells. These morphological responses affirmed the findings of the above-mentioned cell death analysis and are consistent with the analogous findings on WP isolate and blackcurrant encapsulates observed in HepG2 cells, wherein these morphological changes may be tied to the induction of intracellular reactive oxygen species generation, which ultimately results in KB OC cell death (225). Though there are few studies using the combination of AO-EtBr staining assays in oral cancer cells, there seems to be a lack of research applying this specifically to whey-based beverages and in KB OC cells.

6.3.11.6 Comparison of method of action Doxorubicin and T1 WMB Mix

WP contains a high concentration of essential amino acids, especially cysteine, which boosts the production of glutathione (GSH) in cells. GSH is a critical antioxidant that helps neutralize free radicals and protects cells from oxidative damage. While WP enhances antioxidant defences in healthy cells, cancer cells, due to their high ROS levels, can suffer from imbalances when their antioxidant system is disrupted, which leads to cell death. Whey also has immunomodulatory properties that can strengthen the body's immune response against

tumours. Lycopene and βC, abundant in the tomato component of T1 WMB mix, are potent antioxidants known for their anticancer effects. Lycopene inhibits cancer cell proliferation by interfering with the cell cycle, particularly the G1 phase, and induces apoptosis through mitochondrial pathways. It also downregulates key oncogenes like Bcl-2 while upregulating tumour suppressor genes like p53. Furthermore, lycopene reduces oxidative stress, thereby suppressing tumour growth. βC enhances these effects by scavenging free radicals and reducing DNA damage, thus contributing to the reduction of cancer cell viability.

Doxorubicin binds to DNA by intercalating between base pairs, which disrupts the DNA double helix structure. This prevents the proper replication and transcription of cancer cell DNA, leading to inhibited cell division. Moreover, doxorubicin inhibits the topoisomerase II enzyme, which is crucial for repairing DNA double-strand breaks. By stabilizing the topoisomerase II-DNA complex, DOX leads to the accumulation of lethal DNA damage, causing apoptosis in rapidly dividing cancer cells. Doxorubicin stimulates the production of ROS in cells, which damages DNA, proteins, and lipids through oxidative stress. Cancer cells, which are already under high oxidative stress, are more susceptible to additional damage caused by ROS. This oxidative damage contributes to the cancer cell's death by promoting mitochondrial dysfunction and apoptosis. Doxorubicin activates apoptotic pathways by both intrinsic and extrinsic mechanisms. It causes mitochondrial damage, leading to the release of cytochrome c, which activates caspases and triggers programmed cell death. DOX also activates cell surface death receptors, further promoting apoptosis.

While doxorubicin primarily exerts its anticancer effects through direct cytotoxic mechanisms, such as DNA damage, ROS generation, and apoptosis, the T1 WMB mix takes a more nutritional and antioxidant-based approach. The antioxidants in the T1 WMB mix, particularly from whey, tomato, and banana, target cancer cells by reducing oxidative stress, supporting the immune response, and inducing apoptosis. Moreover, while doxorubicin can cause significant

side effects, such as cardiotoxicity, the T1 WMB mix offers a safer, nutritional alternative with fewer adverse effects.

Interestingly, both approaches induce oxidative stress in cancer cells, but through different mechanisms—doxorubicin through ROS generation and T1 WMB mix through antioxidant imbalances. This complementary nature suggests that nutritional therapies like the T1 WMB mix could be used alongside traditional chemotherapy to enhance the overall anticancer effects while mitigating some of the side effects associated with drugs like doxorubicin.

CHAPTER 7
SUMMARY AND CONCLUSION

7.1 SUMMARY

- T1 provides an optimal balance between protein (whey) and other proximate nutrients from tomato, banana, SOC, and FOC. Its moderate whey content (45%) ensures a high protein concentration while maintaining a good balance of other nutrients from the fruit pulps and oil cakes.
- T1 showed superior functional properties, including solubility (79.50±0.70%), wettability, and dispersibility (83.50±2.12%), which enhance its rehydration characteristics, making it more suitable for instant beverage applications. These values are significantly better than the control (C), which has lower solubility (65.50±0.70%) and poorer dispersibility (73.50±2.12%) due to the absence of WP.
- T1 significantly outperformed other formulations in sensory evaluations, particularly in taste (7.45±1.36), texture (7.00±1.30), and overall acceptability (7.42±0.36). This is attributed to the balanced whey concentration that improves mouthfeel, creaminess, and palatability. The control and higher whey formulations (T3 and T4) scored lower due to textural, as well as sensorial issues as the control lacked the creamy texture provided by WP.
- T1 maintains a favourable balance of bioactive compounds from tomato, banana pulp (Vit C, lycopene, βC, TPC), contributing to its higher antioxidant capacity compared to T2, T3 and T4. While the control has higher lycopene content due to its 88% tomato composition, the inclusion of whey in T1 allows it to retain adequate antioxidant potential mostly by its antioxidant bioactive peptides without compromising on sensory properties, which is crucial for consumer acceptability.
- The major minerals in T1 WMB mix, particularly potassium, magnesium, calcium, and phosphorus, contribute to its antioxidant and anticancer activities by supporting enzymatic functions, maintaining cellular homeostasis, and modulating oxidative stress. The analysis

of trace minerals in the T1 WMB revealed significant levels of zinc (Zn), manganese (Mn), and copper (Cu), which contribute to the functional and health-promoting properties of the product.

- The protein quality analyses of T1 WMB mix revealed that the protein present is of superior quality with the presence of essential and non-essential amino acids as well as *In Vitro* Protein Digestibility (IVPD values.
- The amino acid profile of the T1 WMB mix (Table 20) highlighted the presence of essential amino acids that contribute to the potent antioxidant and anticancer properties of WP.
- The *In Vitro* Protein Digestibility (IVPD) assay accounted 86.83 % digestibility of T1 WMB mix when compared against the standard casein protein of 90.09% reflecting a very good digestibility.
- Plastic pouches and metallic pouches offered better protection, especially at refrigerated temperatures, with metallic pouches showing the best performance in limiting microbial growth (lowest counts at 90 days).
- The shelf life of the T1 WMB mix can be considered to be around 90 days, as the product remains organoleptically acceptable and physiochemically stable up to this point.
- WP demonstrated significant cytotoxic potential against KB OC cells, particularly at higher concentrations, with its apoptotic and antioxidant-modulating mechanisms playing a crucial role. As the concentration increases to 250 µg/ml, cell viability further declines to 76.82%. Notably, at 500 µg/ml, a substantial drop in viability occurs, with only 36.84% of cells remaining viable. At the highest concentration of 1000 µg/ml, the viability plummets drastically to 7.86%. This marked decrease in cell viability at concentrations of 500 µg/ml and higher confirms WP as a potent anticancer agent.
- The *in vitro* cytotoxicity data of tomato against KB OC cells underscores the strong anticancer potential of tomato, particularly at higher concentrations, where cell viability

dramatically decreases. At 50 µg/ml, the cell viability is 73.41%, significantly decreasing to 8.73% at 1000 µg/ml, indicating strong cytotoxicity suggesting that higher concentrations of tomato extract have potent cytotoxic effects.

- The results from *in vitro* cytotoxicity data highlighted the significant cytotoxic potential of Nendran banana against KB OC cells, particularly at higher concentrations. With increasing concentrations, there is a significant drop in cell viability, with 75.69% at 250 µg/ml, 21.89% at 500 µg/ml, and 9.85% at 1000 µg/ml.
- *In vitro* cytotoxicity analysis showed that T1 WMB mix had a significant dose dependent cytotoxic potential compared to the individual ingredients at 24 h.
- The *In vitro* cytotoxicity study showed a significant dose dependent cytotoxicity of T1 WMB mix towards KB OC cell line with a low IC_{50} value of 200µg/mL
- T1 WMB mix was found to be safer without any cytotoxicity on 3T3-L1 normal cells, but doxorubicin exhibited cytotoxicity against 3T3-L1 cells.
- T1 WMB mix also induced early apoptosis whereas standard doxorubicin exhibited late apoptosis in KB OC cell line.
- T1 WMB mix displayed G2 phase arrest of 25.93% and S phase arrest of 13.07% in KB OC cells that are nearly identical to G2 and S phase arrest caused by the standard doxorubicin.
- This research study in which developed WMB showcased significant anticancer properties in OC cell line, thereby satisfying the study research question of does whey based multi-ingredient beverage exhibit anticancer properties in OC cell line.

7.2 CONCLUSION

Cancer still remains as an unfulfilled medical necessity globally. The attempt to formulate WMB comprising ingredients that are readily available, less expensive, healthy, and affordable at the same time was fruitful with significant results. The WMB formulation illustrates the presence of bioactive components such as lycopene and βC, along with polyphenols and antioxidant substances of therapeutic value. Additionally, it also exhibited desirable nutritional, sensory, physical, and rheological characteristics, serving as a means of whey valorization. The T1 formulation outperformed the other three in terms of nutritional and bioactive component profiles, as well as sensory attributes. Taken together, the current investigation's findings led to the conclusion that the developed whey-based formulation exhibits promising nutritional properties, including antioxidant and anticancer effects. Further, T1 extract manifested concentration-dependent antioxidant activity that was significant ($P < 0.05$). The MTT assay revealed that a dosage of 200 μg mL^{-1} was effective for 50% cytotoxicity against KB OC cells but had relatively low cytotoxicity on normal murine fibroblast cells (3T3-L1). Further, T1 at this concentration also induced early apoptosis in the same cell line, whereas standard doxorubicin exhibited late apoptosis. In addition, T1 displayed G2 phase arrest of 25.93% and S phase arrest of 13.07% in KB OC cells that are nearly identical to G2 and S phase arrest caused by the standard doxorubicin. It is apparent that phytosignature components in the beverage mix formulation, alone or in combination, may be responsible for the extract's reported anticancer effect. This study thus proposes the prospects of using a novel WMB mix as a functional and supplemental drink targeting cancer disease. But their specific role in cancer prevention or treatment requires further robust research, including clinical trials and mechanistic studies. Discarded whey, flaxseed and sesame seed oil cakes can be effectively valorized into an antioxidant rich, nutritious, WMB mix with bioactivity for therapeutic purposes.

CHAPTER 9

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PUBLICATIONS FROM PH.D. WORK

Title	Journal	Indexation/Impact factor
The effectiveness of whey proteins in prevention and treatment of cancer: a review	Critical Reviews in Food Science and Nutrition Impact factor-11.208	Web of science 11.208
Evaluation of the anticancer potential of whey-based beverage powders using the MTT assay	Journal of chemical health risks	Scopus 0.172 (Islamic World Science Citation Centre)
Valorization of whey into novel instant beverage mix: study of nutritional, physicochemical, rheological and sensory properties	Submitted to journal	-
The antioxidant and cytotoxic potential of a novel whey-based instant beverage mix in oral cancer cell line	Submitted to journal	-

PRESENTATIONS FROM PH.D. WORK

Title	Conference details	Type	Organizers
Formulation and evaluation of anticancer potential of whey based multi-ingredient beverage mix	International Conference on Clinical Nutrition & Dietary Lifestyle Online, 20th – 21st May 2022 Secured **Best Oral Presentation Award**	Oral	Universal Society of Food and Nutrition (USFN)
Valorization of dairy discarded whey, flaxseed and sesame oil cakes into low-cost multi-ingredient beverage mix	28th Indian Convention of Food Scientists and Technologists (ICFOST) Conference Online, 20th – 22nd January 2022	Poster	Association of Food Scientists and Technologists (AFSTI)

APPENDIX I

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
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REVIEW

The effectiveness of whey proteins in prevention and treatment of cancer: a review

Anjana Thampy[a], Meena Kumari Palani Kumar[b,c], Muthukumar Serva Peddha[b,c] and Madhavi Reddy[a]

[a]Department of Clinical Nutrition and Dietetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India; [b]Department of Biochemistry, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India; [c]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India

ABSTRACT

Cancer prevalence is rising rapidly around the globe, contributing immensely to the burden on health systems, hence the search for more effective and selective treatments still remains enticing. Whey, as a natural source, has received extensive focus in recent years because of its intriguing applications to health benefits. Growing consumer appreciation of the nutraceutical effects of whey components makes them an attractive field within cancer research. Whey is a valuable source of superior-quality proteins, lactose, vitamins, and minerals that contribute to proper nutrition as well as help hamper illness and even complement certain disease-related therapy prognosis. As a result, industry leaders and dairy producers are devising new ways to valorize it. Great emphasis on cancer prevention and treatment has been given to whey protein (WP) by the scientific community. WP intake has been proven to induce anti-cancer effects in various in vitro and in vivo studies. Nutritionists and dietitians are now enormously endorsing the role of WP in the therapeutic field, notably for cancer cachexia management. However, human intervention studies with WP are in their infancy and remain to be established with different tumor entities to provide valid proof of its ability to act as a coadjuvant in cancer treatment.

KEYWORDS

Dairy; muscle protein; cachexia; nutraceuticals; therapeutics; supplements

Introduction

Cancer still remains a serious public health problem globally and continues to be a top cause of death throughout the world (Choudhari et al. 2020). Although different treatment approaches have helped significantly increase survival rates over the years, its incidence is still high and so is its mortality. Malnutrition in hospitalized cancer patients is also rising as a burden along with the increasing number of cancer cases (Silva et al. 2015). It has become a serious challenge to oncotherapies as the nutritional status and clinical outcome of cancer patients are linked considerably (Arends et al. 2017; Walsh et al. 2019). All anticancer initiatives aim to reduce cancer deaths and associated malnutrition and promote sustainable strategies for prevention. In view of this, naturally occurring compounds are emerging as novel, vital resources for anticancer therapy.

Whey, considered as a waste product previously, has received tremendous acceptance in the modern health beverage market and therapeutic nutrition sector nowadays. Whey is a by-product of milk curdling obtained during the making of a variety of dairy products, including curd, cheese, paneer, yoghurt, and caseinates, with a wide range of commercial applications (Wherry, Barbano, and Drake 2019). It is made through lactic acid bacteria fermentation, acidification of milk, or enzyme action (Batista, Campos, and Silvestre 2018). It is one of the remarkable and voluminous dairy by-products, that accounts for half of total milk solids (Kumar et al. 2018). Earlier, large quantities of whey were either used as livestock feed or discarded as trash (Macwan et al. 2016). Whey waste disposal represents a substantial loss of potential dietary nutrients and the dairy industry has long struggled to find a long-term use for whey because of the serious environmental and ecosystem threat it poses due to its high biochemical oxygen demand (Wherry, Barbano, and Drake 2019; Rocha-mendoza et al. 2021). Furthermore, the dairy sector suffers a financial setback as a result of many treatment expenditures associated with proper whey disposal. Now, whey has been upgraded to a co-product in the making of dairy foods due to its functional properties (Walzem, Dillard, and German 2002). It is no longer regarded as a waste product, but rather as a treasure trove of nutritionally valuable whey components (Macwan et al. 2016).

The multifactorial cancer cachexia, characterized by negative energy equilibrium along with skeletal muscle atrophy driven by lower dietary intake and metabolic abnormalities, makes cancer associated malnutrition more complex and managing it becomes difficult (Arends et al. 2017). Thus,

CONTACT Madhavi Reddy drmmadhavireddy@gmail.com

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Evaluation of the anticancer potential of whey-based beverage powders using the MTT assay

Anjana Thampy[a], Anbarasu Kannan[b], Thiraviam Vanitha[c], Meena Kumari Palani Kumar[b], Satish Anandan[a], Muthukumar Serva Peddha[b], and Madhavi Reddy[a]*

[a]Department of Clinical Nutrition and Dietetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India;

[b]Department of Biochemistry, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India;

[c]Department of Fruit and Vegetable Technology, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India

*Corresponding author: Madhavi Reddy, Professor, Department of Clinical Nutrition and Dietetics, SDUAHER, Klar

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KEYWORDS
Dairy
Beverages
Nutraceutics
Cancer
Cytotoxicity
Antioxidants

ABSTRACT:

Introduction: Oral cancer is a significant source of mortality and morbidity across borders. Therefore, there isa 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 wasutilized 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.

Conference on CLINICAL NUTRITION & DIETARY LIFESTYLE
May 20th & 21st 2022 | Bangalore, India

CERTIFICATE

OF BEST ORAL PRESENTATION

USFN
UNIVERSAL SOCIETY OF FOOD AND NUTRITION

INTERNATIONAL CONFERENCE ON
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20th & 21st May 2022 | The Chancery Pavilion, Bangalore

This is to certify that **Anjana Thampy** has presented his/her research paper titled "Formulation and Evaluation of Anticancer Potential of whey Based Multi Ingredient Beverage Mix" which has been awarded as **Best Oral Presentation** in "International Conference on Clinical Nutrition & Dietary Lifestyle" Organized by Universal Society of Food and Nutrition (USFN) on 20th & 21st May 2022 at the Chancery Pavilion, Bangalore.

Dr. Pradip Chakraborty
Former Director
Food Safety and Standards Authority of India (FSSAI)
India

Dr. Usha Devi C
Professor & Head
Department of Food and Nutrition
Smt. V.H.D Central Institute of Home Science
India

Universal Society of Food and Nutrition * USFN

Mr. Rudra Bhanu Satpathy
CEO & Founder
Universal Society of Food and Nutrition (USFN), India

CERTIFICATE

OF PRESENTATION

INTERNATIONAL CONFERENCE ON

CLINICAL NUTRITION & DIETARY LIFESTYLE

20th & 21st May 2022 | The Chancery Pavilion, Bangalore

This is to certify that Mr/Ms/Mrs/Dr. ANJANA THAMPY of Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar presented his/her worthy **Presentation (Virtual)** titled Formulation and evaluation of anticancer potential of whey based multi ingredient beverage mix during the "International Conference on Clinical Nutrition & Dietary Lifestyle" Organized by Universal Society of Food and Nutrition (USFN) on 20th & 21st May 2022 at the Chancery Pavilion, Bangalore.

Dr. Pradip Chakraborty
Former Director
Food Safety and Standards Authority of India (FSSAI)
India

Dr. Usha Devi C
Professor & Head
Department of Food and Nutrition
Smt. V.H.D Central Institute of Home Science
India

Mr. Rudra Bhanu Satpathy
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CSIR-CFTRI Campus, Mysuru - 570020, India

on VIRTUAL PLATFORM

POSTER CERTIFICATE

This is to certify that the following Poster was presented at 28th ICFoST organized by AFST(I) HQ in association with its Aurangabad & Mumbai Chapters.

Title: Valorization of dairy discarded whey, flaxseed and sesame oil cakes into low-cost multi-ingredient beverage mix

Presenting Author: Mrs. Anjana Thampy

Co -Author(s): Vanitha T, Meena Kumari Palani Kumar, Muthukumar Serva Peddha, and M Madhavi Reddy

Affiliation: Department of Clinical Nutrition and Dietetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, 563101, Karnataka, India. Department of Fruit and Vegetable Technology; 3Department of Biochemistry, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India

Dr. Vikas Singh Chauhan
Organizing Secretary

Dr. SVN Vijayendra
Chairman – Poster Committee

Dr. Uday S Annapure
Chairman

APPENDIX II

ETHICAL CLEARANCE CERTIFICATE

CENTRAL ETHICS COMMITTEE

Sri Devaraj Urs Academy of Higher Education & Research

POST BOX NO.62, TAMAKA, KOLAR-563 101, KARNATAKA, INDIA

Department of Research and Innovation

Ph:08152-210604, 210605, 243003, 243009, ext. 480. E-mail: co.rd@sduu.ac.in

Central Ethics Committee Re- registered under CDSCO - Registration No. ECR/425/Inst/KA/2013/RR-20 dated 29.4.2020

Central Ethics Committee registered under NECRBHR, DHR - Registration No. EC/NEW/INST/2020/588 dated 28.5.2020

Members

1. **Dr. Kiran Katoch**
   Chairman, Central Ethics Committee SDUAHER, Kolar Ex-Director, National, JALMA Institute for Leprosy & other Mycobacterial Diseases (ICMR), Tajganj, Agra (UP)
2. **Mr. Subramani**
   Assistant Professor Basaweshawara College of Law Kolar
3. **Mr. B. Suresh**
   President - District Chamber of Commerce, Vice Chairman, Indian red Cross Society Reporter Press Trust of India BRM colony Kolar.
4. **Dr. Prakash BG**
   Dean, College of Horticulture, Tamaka, Kolar.
5. **Swami Chinmayananda Avadhuta**
   Co-ordinator, South India Ananda Marga Prachara Sangha Ananda Marga Ashram Kithandur, Kolar (T)
6. **Dr. Prabhu E**
   Professor of Orthopedics SDUMC, Kolar
7. **Dr. N. Sarala**
   Professor of Pharmacology SDUMC, Kolar.
8. **Dr. Sharath B**
   Associate Professor Dept. of Cellular Biology & Molecular Genetics SDUAHER, Kolar
9. **Dr. Shashidhar K N**
   Member Secretary Director, Department of Research & Innovation, SDUAHER, Kolar

No: SDUAHER/KLR/Dept. R&I/ /2021-22 Date: 15.07.2021

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Central Ethics Committee, SDUAHER, Kolar

To:
Mrs. Anjana Thampy
Ph.D. Scholar
Department of Clinical Nutrition and Dietetics
AH & BS, SDUAHER, Tamaka-Kolar-563103

DEPT. OF CLINICAL NUTRITION & DIETETICS * SDUAHER * No. 107 Dt. 28/7/2021 Tamaka, Kolar 563101

Madam,

Subject: Ethical Clearance for Ph.D. Synopsis

The Central ethics Committee of Sri Devaraj Urs Academy of Higher Education and Research, Kolar has examined Ph.D. Synopsis, titled: "**Formulation and Evaluation of Anticancer Potential of Whey Based Multi-ingredient Beverage in Oral Cancer Cell Line**" and the detailed work plan of the project.

The central ethics committee has unanimously decided to approve the project and grant permission to investigator to carry out the research work. The interim and final report has to be submitted to the ethics committee after completion of the project for the issue of Central Ethics Committee certificate. Principal investigator should maintain the records of the Project and consent form for not less than 5 year from the date of completion or termination of the project.

Member Secretary 15/7/2021
(Dr. K.N. Shashidhar)
MEMBER SECRETARY
CENTRAL ETHICS COMMITTEE
SRI DEVARAJ URS ACADEMY OF
HIGHER EDUCATION & RESEARCH
TAMAKA, KOLAR-563 101

Chairman
(Dr. Kiran Katoch)
Chairman
Central Ethics Committee
Sri Devaraj Urs Academy of
Higher Education and Research
Tamaka, Kolar-563101.

APPENDIX IV

CERTIFICATION FOR TOMATO

UNIVERSITY OF HORTICULTURAL SCIENCES, BAGALKOTE
COLLEGE OF HORTICULTURE, KOLAR

Dr. B. G. PRAKASH
M.Sc. (Agri.), Ph.D.
Plant Breeder and Dean,
College of Horticulture,
NH-75, Tamaka (Jack garden)
Kolar-563103.

Phone No.08152-243208
Mobile No. 9480696384
Email:dean.cohkolar@uhsbagalkot.edu.in,
cohkolar@gmail.com

No. Dean office's/COH-K/ 03 /2021-22

Date: 01.04.2021

CERTIFICATION FOR TOMATO

This is to certify that **Mrs. ANJANA THAMPY, Ph.D. Scholar**, from Sri Devaraj Urs Academy of Higher Education and Research, Kolar is doing her research work related to whey beverage formulation using tomatoes of **Arka Apeksha (H-385)** variety which is a high yielding hybrid developed by ICAR-Indian Institute of Horticultural Research, Bengaluru.

University of Horticultural Sciences * BAGALKOT * Office of the Dean College of Horticulture Kolar

PLANT BREEDER AND DEAN
COH, KOLAR

Dean
College of Horticulture
Tamaka, Kolar-563103

APPENDIX V

CERTIFICATION FROM THE FARMER

Date: 11/07/2021

Certification from the farmer

This is to inform that I, Yogananda H.K. purchased Arka Apeksha variety tomato seeds from Indian Institute of Horticultural Research (ICAR-IIHR), Bangalore with the order no 2020-2021/DD9039, dated 11th October 2020 and planted them in my farm and harvested the fruits. Mrs. Anjana Thampy, Ph.D. Scholar from Sri Devaraj Urs Academy of Higher Education and Research, Kolar, contacted me and collected the fruits of Arka Apeksha variety tomatoes for her research purpose.

Yoganda

Yogananda H.K

11/07/2021

APPENDIX VI

Date: 10.12.2021

Certification from the Krishi Officer, Kerala

This is to certify that Mrs. Anjana Thampy, Ph.D. Scholar from Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka had procured and used Nendran variety of banana for her research purpose.

APPENDIX VII

Sensory Score card for sensory evaluation of the product

	Product samples – T1, T2, T3, T4 and Control					
SL. No	**Quality Attributes**	**T1**	**T2**	**T3**	**T4**	**Control**
1	Appearance/Color					
2	Flavor					
3	Consistency/Texture					
4	Taste					
5	Mouthfeel/After taste					
6	Overall acceptability					

Scoring system:

9 – Like extremely, 8 – Like very much, 7-Like moderately, 6- Like slightly, 5-Neither like nor dislike, 4- Dislike slightly, 3- Dislike moderately, 2-Dislike very much, 1- Dislike extremely

Name:

Date:

Comments: Signature

T1

T2

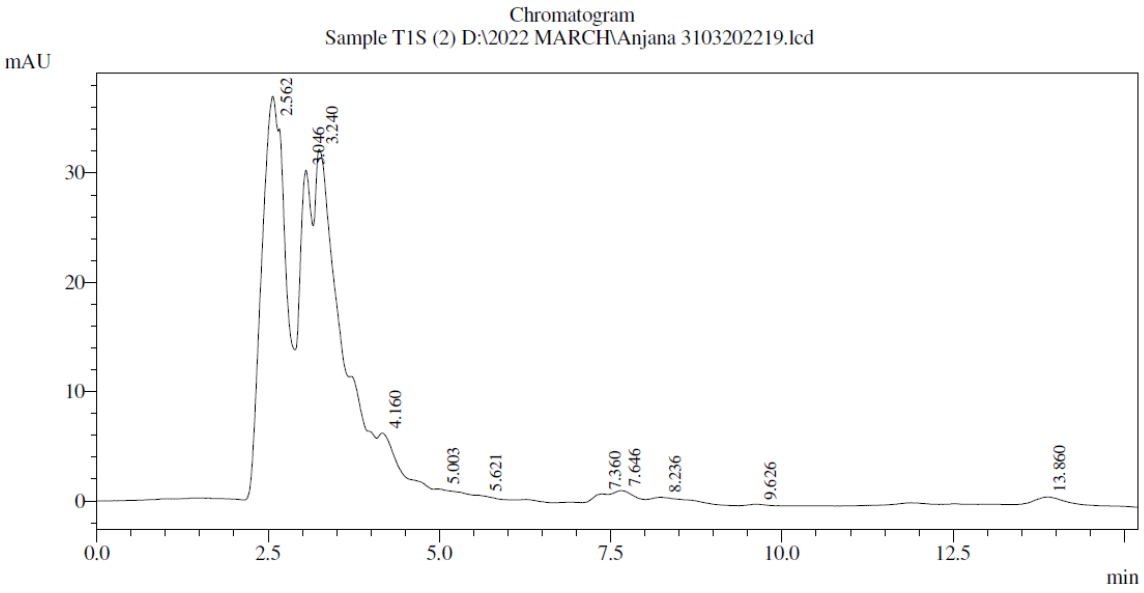
T3

T4

Control

APPENDIX VIII

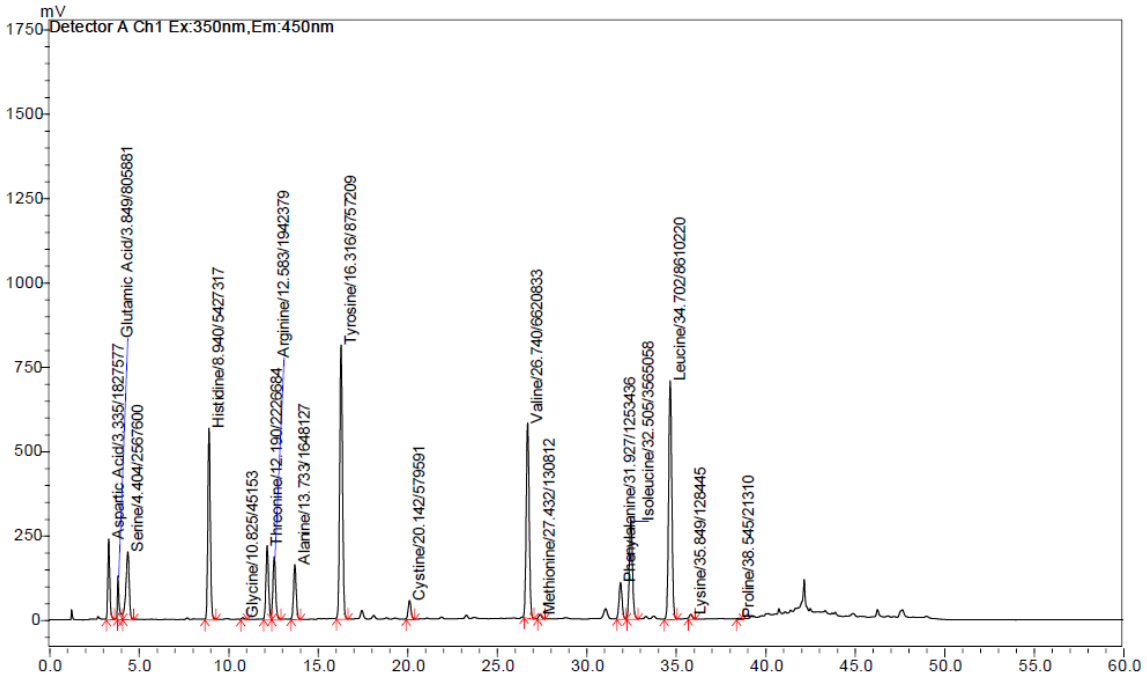
HPLC VITAMIN C CHROMATOGRAM OF T1 WMB mix



APPENDIX IX

UHPLC AMINO ACID CHROMATOGRAM

UHPLC chromatogram of Whey based powdered sample (Code No: 538/T1)



CHAPTER 8

NEW KNOWLEDGE GENERATED

- The attempt to formulate WMB comprising ingredients that are readily available, less expensive, healthy, and affordable at the same time was fruitful with significant results.
- WMB could be employed as a natural antioxidant source with bioactivity for therapeutic purposes, thereby, serving the means of valorization of dairy and agricultural by-products of whey, sesame seeds and flaxseeds oil cake.
- Determined the antioxidant and anticancer potential of WMB mix in OC cell line for the first time to the best of our knowledge.
- Our findings suggest that WMB has anti-cancer properties in oral carcinoma.

LIMITATIONS OF THE STUDY

- Anticancer assays were limited only for initial 24-hours.
- Investigations of animal and human interventional studies were not carried out.

FUTURE LINE OF WORK

- This study can be taken forward by conducting animal and human interventional trials on OC subjects to confirm the efficacy of developed WMB.
- Procedures of the bioaccessibility and bioavailability tests has to be carried out for better clinical utility.
- Further analysis with other cell lines and molecular studies are needed to validate these findings.
- Possibilities of process and product patent filing could be considered.
- Exploring the dissemination of the technologies to self-groups and small-scale industries for microenterprise.

PLAGIARSIM DIGITAL CERTIFICATE

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

Tamaka, Kolar 563103

Certificate of Plagiarism Check

Title of the Thesis/Dissertation	FORMULATION AND EVALUATION OF ANTICANCER POTENTIAL OF WHEY-BASED MULTI-INGREDIENT BEVERAGE IN ORAL CANCER CELL LINE
Name of the Student	MRS. ANJANA THAMPY
Registration Number	20PY4001
Name of the Supervisor / Guide	DR. MADHAVI REDDY
Department	CLINICAL NUTRITION AND DIETETICS
Acceptable Maximum Limit (%) of Similarity (Ph.D. Thesis)	10%
Similarity	9%
Software used	Turnitin
Paper ID	2445577275
Submission Date	05-09-2024

06/09/2024

Signature of Student

06.09.2024

Signature of Guide/Supervisor

University Librarian

University Library
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Head of the Department