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Conclusion should elucidate how the results communicate to the theory presented as the basis of the study and provide a concise explanation of the allegation of the findings.

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The Diet Dynamics in Holy Month of Ramadan

OPEN ACCESS

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Ramadan, one of Islam's five pillars, is a period of frugality from dawn to sunset that lasts a lunar month (29-30 days). In Ramadan, millions of Muslims deepened their relationship with God by fasting, praying and spiritual devotion. Fasting every day before dawn and after sunset, families break their fast together shortly before sunset. Not only is this sacred practice deeply religious, it also provides health benefits that researchers are studying to see how the rhythms of fasting affect our wellbeing. Therefore, it is very helpful in understanding the routine of fasting and nourishment during Ramadan.

Fasting during the whole day means going without food and water for long hours. If sufficient nutrition is not maintained throughout this time, it might result in weakness, dehydration, and a decline in energy levels. The body's normal functioning is greatly affected due to absence of frequent meals. Hence it is essential for individuals to have nutritious foods during non-fasting hours. Many people overeat at iftar and intake high fat and sugar rich foods which causes weight gain and fatigue.

It is essential to consume well-balanced diet at Suhoor that will maintain energy levels throughout the day. Mostly, it is advised to have foods high in complex carbs, proteins such as whole grains, eggs, rice that will keep one satisfied for longer and retain muscle strength. Dehydration can be prevented by the intake of plenty of fluids and eating fruits with high water content such as watermelon etc.

Moreover, A balanced iftar is also recommended to avoid health haphazard. Dates and water have been traditionally used at time of iftar which have both religious and scientific benefits. Individuals should take fiber rich and healthy foods. People should avoid using fried items which will cause digestive discomfort and uneasiness later on. People should maintain proper hydration at night since fasting limits fluid intake during the day.

During this Holy month, the biggest challenge encountered by people is their tendency to overeat at iftar. The extended hours of fasting can lead to increase the appetite for oily and sugary foods which causes indigestion and bloating. Mindful diet plan and portion control can help in maintaining general health and avoiding unwanted weight gain.

Besides spiritual benefits, fasting in Ramadan also have vast physical benefits. It helps in digestion, detoxification and improves health management. According to different studies, fasting boosts insulin sensitivity and metabolic flexibility, which lowers the risk of diabetes and cardiovascular disease. Ramadan diet dynamics focus on mindful eating, water, and balanced nourishment.

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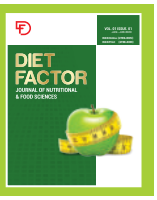

Review Article

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Tech-Driven Evolution of Trait Performance in Oilseed Crops: A Contemporary Perspective

Zohaib Younas[1], Ilyas Ahmad[1], Tayyaba Yousaf[1], Syed Aoun Abbas Kazmi[2], Ahmad Hassan[1], Muhammad Younas[1], Muhammad Imran[1], Ubaidur Rahman[1], Maaz Ahmad[1] and Zia Ur Rehman Mashwani[1*]

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ABSTRACT

Targeted nucleases are resilient genomic tools that accurately modify the intended genome of living cells, regulating functioning genes with great precision. Gene editing techniques (GETs), especially CRISPR-cas9, are utilized for genetic manipulation with greater efficacy, versatility, cost-efficiency, and capacity for high-throughput applications in the fields of medicine, biology, agriculture, and biotechnology. It has been successfully used for the treatment of genetic diseases in humans and oilseed crop improvements such as disease resistance, reducing seed shattering, herbicide resistance, and improving oil quality and quantity. The purpose of this review is to summarize the potential application of GETs to bring improvements to oilseed crops. In the current study, three different methodologies to incorporate desire traits in oilseed crops are discussed, mainly for the needs of farmers and consumer demands. The methodologies included conventional plant breeding (CPB), mutagenesis plant breeding (MPB), and the advanced gene editing tool CRISPR-cas9. Ongoing inventions in the agriculture field and in the last decade (ten years) are focused. Results: Mechanistic representation in detail was given for editing plant genomes using various strategies such as PEG-mediated, biolistic, and agro-bacterium-mediated plant transformation. The modification of agricultural crops was required to increase the nation's economic condition. In the future, to overcome food security issues, researchers from multidisciplinary fields can plan their work in oilseed crops or relevant disciplines for the betterment of humanity.

INTRODUCTION

A precise, robust and efficient genome editing technique (GET), CRISPR-cas-9 has revolutionized molecular biology and genetics [1]. CRISPR functions as a part of adaptive immune system in prokaryotes against invading viruses/phages or plasmid [2]. During encounters, small fragments of invaders DNA (spacer sequences) become part of bacterial own's genome known as a CRISPR locus. The bacteria then transcribe the Crispr locus along with newly spacer sequences into mRNA (tracrRNA). Multiple spacers and repeats are interspersed within the CRISPR array to produce a single precursor RNA molecule known as pre-crRNA. RNA polymerase is typically responsible for this transcription. Pre-crRNA is processed by RNAase III by binding at spacer sequences, which cleaves it into gRNA. The sgRNA is generally composed of a 20-bp sequence unique to the target DNA, followed by a brief "NGG" or "NAG" sequence known as the "PAM," which is required for Cas9 protein interaction [3]. Cas9 associates with the guide RNA molecule, typically a synthetic single-guide RNA (sgRNA) that merges the roles of both crRNA and tracrRNA, to

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create the Cas9 complex (Figure 1). Most notably, CRISPR-associated proteins like Cas9 and Cas12a nucleases have been responsible for the fast expansion of genome editing [4]. Addressing more obscure CRISPR-Cas systems in bacteria and archaea is critical because they have the potential to significantly expand the scope of plant gene editing tools [5]. Implementing genetic modifications such as substitutions, insertions, and deletions which improve agronomic features can speed up crop enhancement and breeding efforts [6]. The phylogenetic tree for CRISPR-Cas was categorized into two major classes (1 & 2) along with six types and into 16 subtypes. Cas9 and Cas12a/Cpf1 are most common well-known nucleases used for plant genome medication belongs to class 2 with type II and IV, respectively [7, 8]. Additionally, the CRISPRa (CRISPR activation) and CRISPRi (CRISPR interference) techniques rely on Cas9 with inactivated cutting capabilities (dCas9) coupled with various effector domains to regulate the transcription of target genes. At specific target sites, the CRISPR/Cas9 system induces double-stranded DNA breaks (DSBs). After the DSB is repaired, genetic changes may ensue due to the repair machinery of the cell. Cell-specific DNA repair mechanisms, including the frequently error-prone non-homologous end joining (NHEJ) and the less commonly occurring homology-directed repair (HDR), play a crucial role in fixing double-stranded breaks (DSBs) during genome editing [9]. NHEJ is typically utilized to modify genetic sequences, while HDR can introduce or modify information at a specific genomic locus using carefully designed repair templates [10]. Brassica napus L. (2n = 38) has evolved from ancient interspecific hybridization techniques with two diploid species, Brassica rapa (2n = 20) and Brassica oleracea (2n = 18) [11]. The existence of multiple gene copies with high sequence homology in canola (Brassica napus) complicates the gene function research. If one wants to create a consistent phenotype, it is critical to knock off all homologous genes [12]. Recently, the versatile CRISPR/Cas system has gained prominence for efficiently introducing mutations at multiple gene sites in multi-copy gene knockout studies [6]. Additionally, it was found in literature that genome editing has been successfully used for over 40 species of crops in 25 countries, having the ultimate objective of improving agronomy, food and nutritional quality, and resistance to abiotic stress [13]. Despite its enormous assurance, still six genome-edited crops traits have gotten commercial clearance .

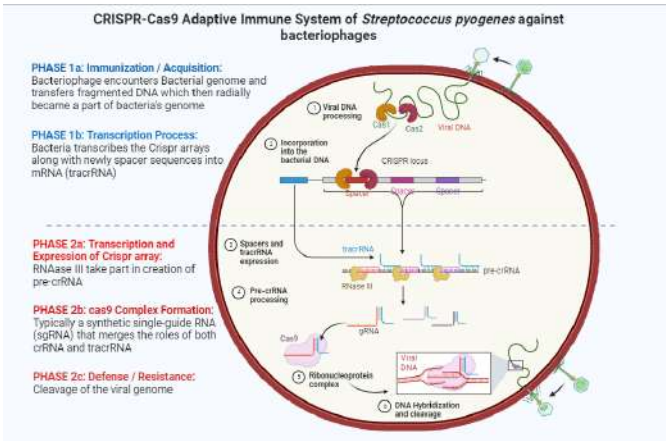

Figure 1: Bacteria Shows Acquired Immunity Against Viral Genome

Application of CRISPR/cas9 in Genome Editing

Over time, the research of gene function has commonly used approaches such as overexpression, silencing, and DNA insertion, all of which necessitate the incorporation of foreign DNA segments into the host genome [14]. Yet, problems occur during plant genetic transformation, especially the danger of unintentional insertions, which lead to regulatory restrictions on transgenic crops as genetically modified organisms. These legislative constraints and potential unforeseen genomic changes impede the progress and utilization of such crops [15]. Researchers are increasingly enthusiastic about genome editing techniques (GETs) owing to their remarkable attributes, including precision, versatility, cost-efficiency, and capacity for high-throughput applications. These GETs have become invaluable for the functional characterization of specific genes in a diverse range of organisms, such as bacteria, plants, animals, and even humans [16-18]. The limitations of GETs extend beyond plant genome modification, as they have proven successful in modifying gene traits in a variety of organisms, including fruit flies, mice, rats, and even in the treatment of human diseases (Figure 2). The error prone changes in hematopoietic stem cells responsible for the cause of hematologic diseases [19, 20]. Successful case studies utilizing CRISPR are reviewed individually [21, 22]. The application of CRISPR/cas9 for genome editing in diverse range of fields as depicted in Figure 2. Contribution of cas9 in biomedical field; A landmark study used CRISPR/Cas9 to repair cataracts in a rat model caused by base deletions. They co-injected mRNA expressing Cas9 and a sgRNA into the fertilized eggs of mice prone to cataracts in this work. The experimental trial was performed on twenty mouse progenies, in which ten mice (45.4%) contained mutant allele. Despite these six mice (27.3%) displayed NHEJ-mediated insertions and deletions (indel) and four (18.1%) underwent HDR-mediated repairs. Surprisingly, all four mice with cataracts fixed by

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HDR induction were entirely healed, as were two of the NHEJ-induced animals. These studies proved CRISPR/Cas9's ability to change the genome for the therapy of hereditary disorders [23, 24]. Another praising contribution to this cas9 tool in field of medicine. Human beings suffer from genetic disease can be cured with CRISPR-cas9 application. It was revealed that (GETs) is a potential contender in treatment of numerous genetic, bacterial and viral diseases. CRISPR-Cas9 offers a very promising method for treating hematological disorders by targeting the HBB gene. Hematopoietic stem and progenitor cells (HSPCs) and induced pluripotent stem cells (iPSCs) derived from patients have shown success with this technique. HBB gene alterations were corrected in these trials, resulting in lower hemoglobin levels and less sickle cell disease. The clinical application of CRISPR-Cas9 in hematological disorders shows its therapeutic promise [19]. Finally, use of CRISPR-cas9 system was addressed for integrating plant genome especially focused on oilseed crops: To address concerns regarding erucic acid in canola oil and its potential health effects; Shi et al., conducted research aimed at reducing erucic acid levels in Canola plants [25]. Using the B. napus cultivar "CY2" as the transgenic recipient, they modified fatty acid compositions by introducing a BnFAE1 fragment driven by napin A promoters and co-culturing hypocotyls with Agrobacterium tumefaciens EHA105. Through seed-specific knockdown of BnFAE1 in CY2, the researchers effectively changed the fatty acid composition, notably reducing erucic acid to less than 3% in the resulting transgenic canola lines. This intervention involved RNAi constructs that successfully interfered with BnFAE1 mRNA levels in F1 hybrid seeds.

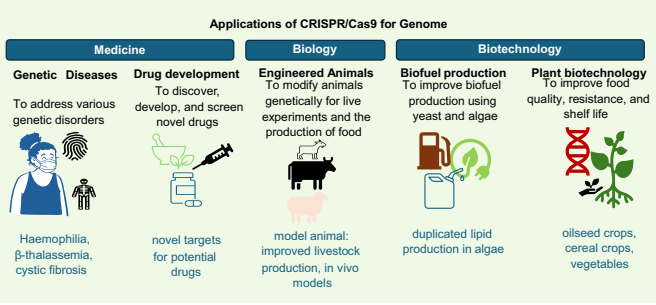

Figure 2: Pictorial Representation of CRISPR/Cas9 Applications in Field of Medicine, Biology and Biotechnology

Biochemistry and De novo Synthesis of Fatty acids (FAs)

It has been described that genes are responsible for controlling oil/seed trait in *Brassica napus*. They developed transgenic depressed lines in which BnFAD2 and BnFAE1 were targeted through using napin A promoter and succeeded. The de-expression of both FAD2 and FAE1 resulting in increment of oleic acid unsaturated fatty acid (OAUFA) and protein profiling in seeds. They also determined that increase in OAUFA is inversely proportional to erucic acid (EA) content. EA content lower in seed of *Brassica napus* when OA content was increased, it improved overall oil nutritional profile. Furthermore, the poly-unsaturation in oleic acid (18:1) to linoleic (C18:2), linolenic (C18:3) enhanced its stability. Meanwhile, the protein content is a major contributor to the meal energy value for feed. The formation of complex molecules such as FAs from precursor units i.e. (sugars and amino acids) referred to as de novo synthesis. The oil is categorized into two main types (1) consumable and (2) non-consumable. In mentioned categories the oil that obtained from *Brassica napus* is not directly consumable before it undergoes through several analysis and process. The seed oil is predominately composed of TAGs, which are essential for human nutrition. To check the detailed study about FAs synthesis, it occurred in two different organelles such as plasmid and cytoplasm. Acetyl-CoA is the precursor unit for UFA synthesis which undergoes through series of reactions resulting in various intermediate compounds. All the reactions are carried by specific enzyme [26]. The FAs synthesis is driven by Acetyl-CoA carboxylase enzyme, in which acetyl-CoA is converted to first intermediate compound malonyl-CoA. The malonyl-CoA is then converted into other unstable intermediate compound known is stearic acid in presence of ACP. The ACP is responsible for the addition of two carbon groups into newly synthesized compound. Furthermore, the synthesis of FA proceeded and resulted in the formation of oleic acid. The FAB2 enzymes drive the reaction which occurred in plastid of plant cell. Despite this, the chain elongation formation in endoplasmic reticulum or cytosol of plant cell depicted in figure 3. The oleic acid is then converted into either eicosenoic acid synthesis in the present of fatty acid elongase (FAE1) or linoleic acid with the help of fatty acid dehydrogenase (FAD2).

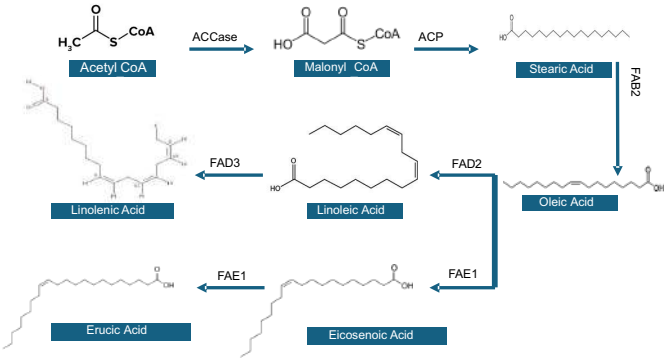

Figure 3: Mechanism for Fatty Acid Synthesis in *Brassica napus* L. The Synthesis Process from Acetyl-CoA to Oleic Acid Occurs in Plastid and Elongation Occurs in Endoplasmic Reticulum

Traditional Breeding Techniques (TBT)

The inherent genetic variety found in crop cultivars,

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landraces, and their wild relatives offers the genetic diversity that's needed for plant breeding and agricultural development. Molecular research elucidated that genes play a critical role in casting plant attributes including shoot branching, tiller count, flowering period, grain production, grain size, nutrient utilization efficiency, and tolerance to both environmental and biological challenges [27, 28]. In the face of environmental concerns, shrinking farmlands, and depleting groundwater resources, plant breeding innovations are crucial to boost crop yields and establish resilient agriculture to meet the consumer demands. In modern-day farming, key crop-enhancing methods such as conventional plant breeding (CPB) or hybridization, mutation breeding (MPB), and transgenic techniques are indispensable [29]. In this review, three different strategies are discussed such as 1) Conventional plant breeding (CPB), 2) Mutagenesis plant breeding (MPB) and 3) Transgenic using CRISPR-cas9 system. Traditionally, CPB has been used in agriculture to develop new plant varieties by selecting and crossing parent plants that exhibit desired traits [30]. Using this technique, plants with certain qualities, such as disease resistance, higher yields, and better yield quality are intentionally pollinated together. It is frequently most laborious and time-consuming method. This repeated selecting and backcrosses cycle tries to produce new plant varieties with an assortment of beneficial traits that meet the demands of consumers, farmers, and changing environmental circumstances [31, 32]. It is concluded that CBP takes around a ten-year period to introduce favorable alleles while optimizing polymorphism effectiveness through genetic recombination. Interestingly, unlike genetic modification procedures, CBP depends on natural genetic diversity and does not entail the incorporation of foreign genes into the plant's DNA. Secondly, mutagenesis plant breeding is a technique used in agriculture to induce genetic variations in plants by exposing them to physical mutagenic agents such as radiation or chemicals. Recently, research induced mutation using various doses of irradiation ranges from (25 to 300 Gy) heavy ion beam (HIB). There is limited research on the mutation features caused by various HIB dosages employing low-generation (M1 - M2) mutants without phenotypic bias [33]. They proceeded with their experiment up to 6th generation and concluded that M3 and M6 have highest number of phenotypic mutants [34, 35]. Based on research, mutagenesis plant breeding takes years to produce mutant plants with desirable traits. It would be noted that during mutagenesis, personal care must be kept to preference and protective kits should be used to protect oneself from HIB. Finally, the CRISPR-cas9 system, a novel tool of gene editing utilization for crop improvement, got fame due to its success rate, less off-targeting effect and high precision rate. Interestingly, gene editing tools might help to accelerate the process [36]. Braatz *et al.*, revealed that CRISPR/Cas9 was initially used to improve shattering and disease resistance in canola by targeting a particular gene (BnALC) [37]. Extensive research (2017 to current day) has proved the maturity of technology, promoting substantial improvements in Brassica napus breeding while offering an understanding of gene function, molecular processes, and prospective routes for seed oil enhancement [38, 39].

CRISPR/cas9 Delivery Platform

Current plant genome editing techniques (GETs), based on established plant transformation methods are presently limited to a few species. It may sound easier to transport CRISPR-cas9 systems into rigid cell structures, but it is quite challenging [40]. Plants face challenges with DNA template delivery, limiting nuclease-initiated homology-directed repair (HDR). Further, the researchers faced difficulties in identifying and successfully retrieving targeted plants. To overcome this, they utilize CBEs and ABEs, enabling specific transitions but not transversions, insertions, or deletions. Moreover, transgene-free altered plants must be produced for commercial reasons, which necessitates reagents and methods of delivery that are DNA-free. Plant cells can be transformed using PEG, Agrobacterium, bombardment, or biolistic methods [41, 42]. The mechanistic approaches for delivery of CRISPER-cas9 system or reagents into plants are given below in detailed (Figure 5).

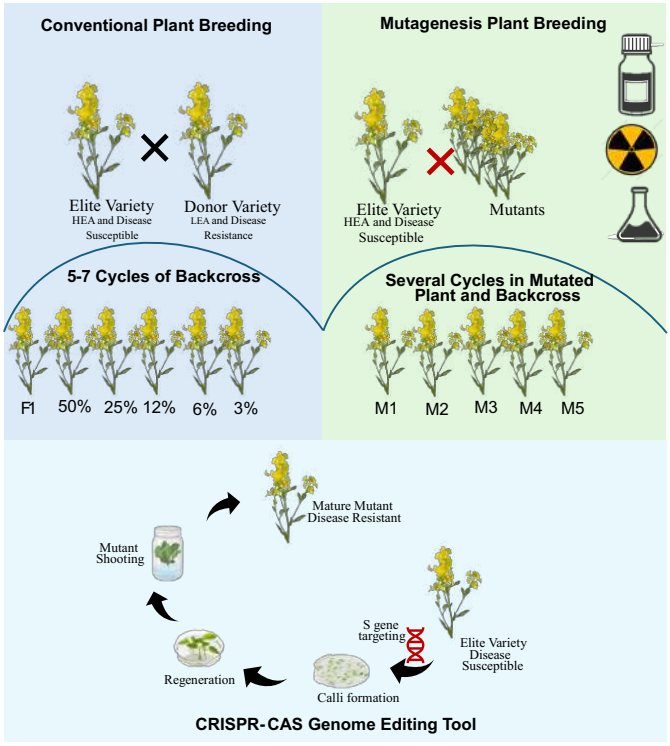

Figure 4: Breeding Techniques for Oilseed Crop Improvement

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PEG Mediated Delivery of CRISPR Reagents

Plant, fungal, bacterial, or archaeon protoplasts are cells that consist of their cell walls which are destroyed by plasmolysis, retaining just protoplasm and plasma membrane [43]. The isolation of protoplasts increases their susceptibility to gene delivery methods. The DNA, RNA, RNPs incorporate into host targeted sites or cell wall free protoplast transports with help of nanocarriers [44]. The main issues with PEG-mediated transformation are that it may cause toxicity or cytotoxicity. To tackle this issue, various transformation methods like electroporation, lipofection, and biolistics have been proposed [45]. Protoplast transformation for genome editing is constrained by explant type, protoplast quality, and the fragility of protoplasts, necessitating careful handling. A key challenge in PEG-mediated transformation is setting up optimal conditions for explants, culture types, and light exposure. It was difficult for researchers to reduce oxidative stress during isolation and culture. The incorporation of CRISPR elements into plant cells, notably oilseed crops, is a hot field of study with the goal of improving agricultural attributes like production, disease resistance, and nutritional value. Designing markers associated with regeneration and proto-clonal variation could be based on factors like chromocenter (re)assembly, ROS activity, DNA methylation, histone methylation, phytohormone ratios, or gene expression. This approach has the potential to create custom and genotype-specific regeneration protocols, ensuring the broad applicability of protoplast-based techniques.

Biolistic Transformation

Biolistics or particle bombardment is a prevalent technique used to transform plants that are resistant to Agrobacterium infection. This technique involves propelling gold or tungsten particles (0.6 µm and 1.0 µm) coated with DNA at high speeds into plant tissue, allowing the DNA to enter plant cells [46]. Reagents are typically applied to microcarriers in an aqueous solution and subsequently precipitated using chemicals such as spermidine/CaCl2/PEG, glycogen, or a cationic lipid reagent. It is extensively used for delivering plasmid DNA, ssDNA, RNA and (ribonucleoprotein) RNPs into chloroplasts and mitochondria. Once inside, the DNA, RNPs separates from the particles, leading to transient expression or stable integration into the host genome. In one work, Luo *et al.*, administered TALEN proteins, ALS2T1L and ALS2T1R, to a region 306 bp downstream of the NbALS2 genes in N. benthamiana protoplasts resulting in a 1.4% mutation frequency. Unlike Agrobacterium, biolistics physical DNA delivery bypasses host-range limitations. The main disadvantages of delivery include uncontrolled integration at many genomic loci when delivered as DNA, laboriousness, and embryo rejuvenation. A significant issue with this tool was found to be the inability to control bombardment sites when targeting organelles like the cytoplasm, nucleus, mitochondria, and plastids [46]. A dual-barreled gene cannon has been created alongside cell counting software to standardize bombardment tests. By adding an internal standard, therefore reducing the usual deviation between bombardments. These improvements attempt to increase the consistency and accuracy of gene delivery systems [47]. It has effectively been delivered to various crop plants such as canola, corn, cotton, soybean, and wheat. This method offers a versatile approach for successful genome editing across multiple plant species [48]. Standardized growth mediums, tissue culturing, vector designing, Selectable markers, gene of interest, Agrobacterium medium plant transformation, mutants' generation all steps carefully needed for a successful outcome. It is summarized that, no doubt these gene editing tools may save time but also lead to productive outcomes also.

Table 1: Biolistic Method for CRISPR/Cas9 System Delivery in Oilseed Crop

Oilseed Crops	CRISPR-cas9 Vector	Selectable Markers	Targeted Genes	Targeted Outcomes	References
Glycine max	QC799 and RTW831	Pat	ALS1	Indels, Rep (HDR) & Editing	Ran *et al.*, 2017
Glycine max	QC810 and RTW830, QC799 and RTW831	*HptII*	DD20, DD43	Indels, Rep (HDR)	Li *et al.*, 2015
Brassica napus	pP1C-4	-	GhCLA1, GhVP	Indels, Rep (HDR)	Chen *et al.*, 2017
Gossypium hirsutum	pCAMBIA-1300	hygromycin	FAE1, FAD2	Indels, Editing	Shi *et al.*, 2022

Agrobacterium-Mediated Plant Transformation

Genetic transformation techniques have advanced crop improvement by integrating new genes, fulfilling the demand for high-yield, quality crops with features such as enhanced oil production, herbicide tolerance, and disease resistance. Agrobacterium transformation remains key for delivering gene-editing tools like CRISPR/Cas variants, base editing, and prime editing into plants. This transformation technique may process through various steps. Initially, research initiated with identification of the specific genes within the plant genome that are to be modified using CRISPR/Cas9. This step proceeded after as development of the CRISPR/Cas9 constructs that carry the guide RNA (gRNA) targeting the chosen gene(s) and the Cas9 nuclease. Furthermore, incorporation of the designed CRISPR/Cas9 constructs into Agrobacterium tumefaciens, a common method to transfer genetic

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material to plant cells is mandatory. The tricky step is following by incubation of the Agrobacterium tumefaciens containing the CRISPR/Cas9 constructs with plant cells to facilitate the transfer of the genetic material. Selection of plant cells that have incorporated the CRISPR/Cas9 constructs and initiating the regeneration process to grow these transformed cells into whole plants [49].

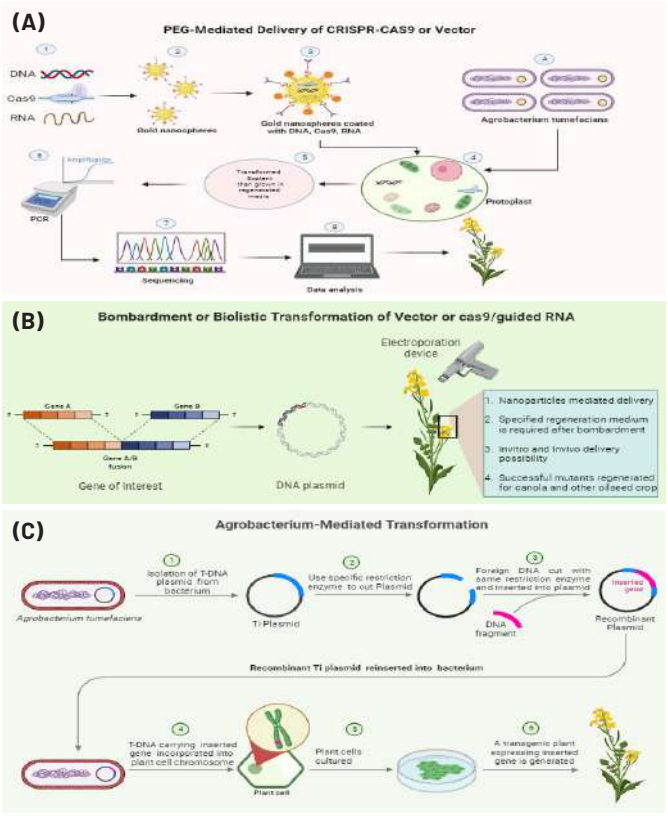

Figure 5: The mechanism for CRISPR/cas9 system delivery platforms using PEG-mediated, Biolistic and Agrobacterium mediated transformation. A) In PEG mediated CRISPR-cas9 delivery onto gold coated nanospheres was carried with gene gun and transferred into protoplast via PEG. In process proceeded to delivered this in newly explant formation and then placed these explants in newly prepared regeneration medium for shooting. Then the PCR was done to multiply the desired sequences and sequencing will be performed. The data analysis regarding this will be visualized on screen for confirmation. B) In biolistic mechanism, the gene of interest along with cas9 is carried out in vitro and then incorporate into protoplast. The foliar targeting with nanoparticles mediated delivery of cas9 should be carried out using gene gun directly. C) The step wise Agrobacterium mediated plant transformation is described well in schematic representation.

Modification in Oilseed Crop by Base Editing and Prime Editing Utilization

"Base editing" offers a precise method for changing nucleotides without disrupting genes or needing donor templates. Base editors offer a platform to change one base to another facilitated by the cytosine or adenosine deaminase domain. The cytidine deaminase enzyme transforms cytosine into uracil by removing an amino group, creating a U-G mismatch. DNA repair pathways then resolve this mismatch by forming U-A base pairs (Figure 6). Following this, a T is added to the new strand, leading to T-A base pairs, causing programmed C-G to T-A conversion. In 2016, Harvard University researchers, led by David Liu, developed BE1—a base editor combining a rat APOBEC1 cytidine deaminase enzyme with a dCas9 using a 16 amino acid XTEN linker [50]. This method allows for single-base modifications, potentially leading to beneficial trait variations in agricultural plants, thereby aiding in crop development. By rectifying single-base modifications or single nucleotide polymorphisms (SNPs) without disrupting the gene, the base-editing technique minimizes indels [51]. It has been revealed that Canola has employed base editing strategies like CBE and ABE. Herbicide-resistant canola has been produced using CRISPR/Cas9-mediated CBE, which successfully introduces C to T conversion in canola sgRNA targets, simplifying weed control for canola production [52]. This efficient method shows promise in establishing new characteristics in crucial agricultural crops, greatly contributing to food security. The primary editor has found extensive use in cereal and vegetable crop breeding research, yet its application in oilseed crops has been limited [53]. This limit is set because our study primarily aimed to gather optimal information on utilizing the CRISPR-Cas9 system for oilseed crop advancement.

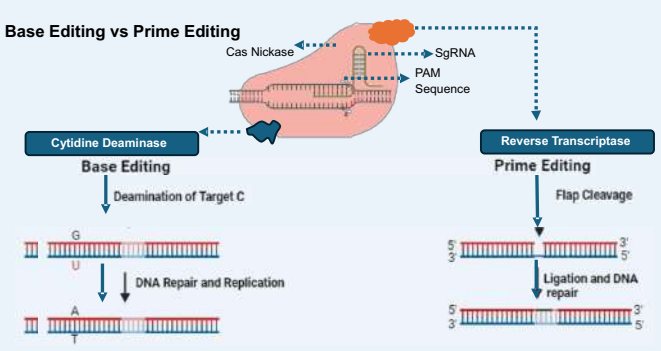

Figure 6: Pictorial Representation of Genome Editing through Base Editing and Prime Editing Utilization for Oilseed Crop Modification

Ethical Concerns in GETS (CRISPR/cas9)

The ability of gene editing technologies like CRISPR-Cas9 to modify the genetic makeup of living organisms, including humans, animals, and plants, has raised several ethical issues. There are many ethically concerns related to this CRISPR/cas9 system described as; Firstly, Off Target Effects: These off-target effects might have unanticipated

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health ramifications in people receiving CRISPR-based medicines. The major concerns related to humans might be later it poses a risk of genetic mosaicism [54-56]. Secondly, Germline Editing: It is extremely ethically problematic to edit the germline, the genetic material that is passed on to future generations. It raises concerns about "designer babies" and genetic enhancement if one alters a person's germline. The third is Environmental Impact: Changing in the genetic makeup of plants results in bioethical issues such as disruption of ecological balance [57]. Fourth is related to Access and Equity: A study proposed issues regarding fair accessibility to genome editing technology. Will genetic improvements be available primarily to the vibrant, resulting in inequities and societal divisions? Moreover, number five is linked to Dual Use Concerns: There is a risk that the same technology used for beneficial purposes could be misused for nefarious purposes, such as creating bioengineered bioweapons. Finally, Genetic Diversity: Genome editing can reduce genetic diversity if used on a large scale, potentially making populations more vulnerable to certain diseases and reducing adaptability [58].

Future Perspective of CRISPR/cas9 Techniques

Plant genome editing will benefit from the use of CRISPR/Cas due to its multiplexing, high throughput editing, and ability to rearrange chromosomes and modify epigenomes. Despite their ease of introduction into plant organelles, Cas nucleases and gRNAs cannot yet be used to edit plastomes and chondriomes. The ongoing study on oilseed crops strives to offer a fresh opportunity for upcoming researchers to contribute to this field. Till now, there are no soyabean varieties which show resistance to abiotic stresses. Likewise, a research gap exists in the realm of hybrid canola varieties, which confront challenges concerning elevated erucic acid levels and undesirable glucosinolate composition with anti-nutritional properties. Moreover, Cotton is a water-intensive crop, and its cultivation often exacerbates water scarcity, especially in regions where water resources are limited. Developing a drought-tolerant cotton variety has the potential to address this problem. Achieving this could involve inducing mutations in the genome to integrate the desired gene of interest via agrobacterium-mediated plant transformation. Further, the CRISPR/Cas9 system has great potential to target cancer causing viruses and genetic disorders. "Last but not least" CRISPR system has significantly influenced cancer research and is poised to maintain an indispensable role in the times ahead.

CONCLUSIONS

New technologies often replace traditional ones due to their higher success rates in clinical trials or ongoing experiments aimed at addressing these concerns. Traditional plant breeding requires several years to achieve desired goals related to food security and environmental concerns, such as developing traits like herbicide resistance, drought resistance, salt soil tolerance, and resistance against infectious diseases. Moreover, when feasible, the commercialization of products or food supplies includes the adoption of advanced techniques within their specific domains. Recently, all of this has become achievable through the implementation of the CRISPR/Cas9 system. In fundamental research, CRISPR/Cas has proven invaluable, especially in gene targeting, knockouts, and gene expression control.

Authors Contribution

Conceptualization: ZURM
Methodology: ZY, TY, SAAK, MY, MI
Formal analysis: IA
Writing review and editing: ZY, IA, TY, AHM MY, MI, UR, MA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Assessment of Stunting and Its Associated Factors in Children Under 2 Years of Age in Tehsil Umerkot

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ABSTRACT

Stunting, a key indicator of chronic undernutrition, remains a critical public health challenge in Tehsil Umerkot, Pakistan, reflecting underlying socioeconomic and nutritional deficits. **Objective:** To find stunting in children under 2 years old and its association with sociodemographic variables in Tehsil Umerkot Pakistan. **Methods:** A cross-sectional study was conducted at urban Tehsil Umerkot, Pakistan. A total of 385 respondents were selected from 4 different public healthcare facilities of District Umerkot, through a simple random sampling technique. SPSS version 26.0 was utilized for data entry and analysis. An anthropometric assessment was carried out for the children and height for age was calculated. All children with a z-score below -2 S.D of WHO standards were considered stunted. Pearson Chi-Square was used to investigate the variables linked with stunting. **Results:** Among the 385 respondents, the majority of the children were female (n= 202, 53%). Mothers of the majority of the children were illiterate (n= 293, 76%). It was found that the majority of the children were stunted (n=360, 96%) out of which 8% (n=30) were severely stunted. Breastfeeding, duration of exclusive breastfeeding, and fruit and vegetable consumption were found statistically significant with stunting (p value<0.05). **Conclusions:** The majority of the children under 2 years of age in Umerkot were stunted. Various factors were identified that play a protective role against stunting among them exclusive breastfeeding, and fruit and vegetable consumption were important. Routine nutritional assessment and interventions are needed to prevent stunting in Pakistan.

INTRODUCTION

Stunting is a malnutritional condition in which a child's growth is hindered and is not according to his or her age. It affects nearly 22% of the children in the world [1]. Stunting causes a huge burden of physical, psychological, and social problems. It is also responsible for the poor quality of life of the individuals [2]. Previous studies found that stunting is associated with long-term consequences which include poor academic achievement and productivity loss in adulthood [3]. Stunting is a complex issue resulting from inadequate in-utero nutrition, childhood illnesses, poor maternal health and nutrition, micronutrient deficiencies, low socioeconomic position, and insufficient feeding habits for infants and early children [4]. Stunting frequently begins in utero and correlates with inadequate breastfeeding and supplemental eating habits; hence, the period from conception to the second birthday is crucial for preventing the adverse consequences of malnutrition [5]. Other risk factors include socioeconomic inequalities, household food insecurity, geographical differences, childhood morbidities and maternal literacy [6]. Furthermore, stunting is also associated with poor sanitary conditions, improper hygiene, and indoor air pollution [7-9]. Other factors associated with stunting among under-fives are gender, age, birth order birth size, and Low Birth Weight (LBW) of children [10]. Stunting has serious health consequences throughout life. This condition has the potential to result in significant difficulties at delivery, decreased cognitive capacity and development, absence from school, and poor social and emotional skills. Furthermore, chronic illnesses are more prevalent in

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advanced age, resulting in elevated healthcare expenditures [11]. Consequently, the prevention of stunting has been established as one of the six primary global nutrition objectives for the period leading up to 2025 [12]. Studies indicate that the job output of stunted youngsters is inferior to that of their non-stunted counterparts [3]. Pakistan's elevated malnutrition rates (40.2% stunting, 28.9% underweight, and 17.7% wasting) signify a persistent child nutrition problem [4]. These levels of malnutrition position Pakistan as the second-highest burdened country in the region, behind India. Although progress has taken place and the proportion of underweight children under 5 years has declined from 32% to 28.9% between 2011 and 2018, during the same period stunting has decreased from 44% to around 40.2%. Improving uptake of maternal and early childhood health services thus becomes the first and perhaps most critical step towards improving human capital in Pakistan. Various studies have been carried out in Pakistan to highlight this crucial issue. However, the literature is scarce regarding the nutritional status of children in remote areas of Pakistan. So, to fill this gap, the current study was carried out in a remote area of Pakistan, Umerkot. Umerkot is a small tehsil in Sindh, Pakistan where the majority of the people are illiterate and belong to lower socioeconomic status. The district has access to only one functioning Nutrition Stabilization Centre at the District headquarters in Umerkot.

The current study was intended to identify the levels of stunting among children under 2 years of age in Umerkot and highlight different sociodemographic, healthcare, and environmental factors associated with stunting.

METHODS

It was a cross-sectional study, carried out from March 2022 to August 2022. Geographical selection was done by using the random sampling method. A list of public health facilities, District headquarters hospitals, and Rural and basic health centres were selected based in urban locations of Tehsil Umerkot for the collection of data. The study was carried out at different public healthcare facilities in District Umerkot. A total of 4 health facilities were selected for data collection. From the list of Lady Health Supervisors (LHS) and Lady Health Workers (LHWs) attached with the randomly selected health facilities, randomly select the LHS and the LHWs in the study [4, 12]. The study population consisted of children aged 6 to 23 months who lived in the study region and were picked at random. Data were collected from their mothers/caregivers regarding the feeding practices of children, healthcare facilities, and environmental factors. Respondents to assess their nutritional status by taking their anthropometric measurements (weight, length /height, MUAC, and Oedema). In order to get the sample size, the OpenEpi, Version 3.01 software's proportional formula for sample size computation was used. The previous prevalence of stunting among children was taken as 39.4% [6]. The calculated sample size was 367 with a 95% Confidence Interval (C.I.) and a 5% margin of error. After adding a 5% non-response rate, the final sample size came out to be 385 children. Data were collected using an interview-administered questionnaire. The length/height, weight, Mid-Upper Arm Circumference (MUAC), and oedema of the children under research were measured using medical-grade equipment from SEECA. Each kid was measured three times to obtain the precise reading. The height for age was tested against WHO-provided guidelines to assess the levels of stunting among the study population. Data were analyzed using SPSS version 26.0. Children were divided into two groups: stunted and non-stunted based on study findings. The Pearson Chi-Square test was employed to examine the connection between stunting and sociodemographic factors. Study was conducted after taking ethical approval from the IRB committee of Al-Shifa School of Public Health Rawalpindi (IRB No. MSPH-IRB/13-36).

RESULTS

In total, there were 385 people who signed up to participate in this study. The majority of the respondents were girls (n=202, 52.5%). Mothers of most of the children were housewives (n=333, 86.5%) and were illiterate (n=293, 76%). Demographic features of the children and their mothers are shown in table 1.

Table 1: Sociodemographic Characteristics of Under 2-Year Children and Their Mothers (n=385)

S. No.	Variables	Frequency (%)
Age of the Child		
1	6-10 Months	88 (23%)
	11-17 Months	172 (45%)
	18-23 Months	125 (33%)
Birth Order		
2	1-3	279 (73%)
	4-7	104 (27%)
	>7	2 (0.5%)
Gender		
3	Male	183 (48%)
	Female	202 (53%)
Family Monthly Income		
4	<15,000 Rs/-	202 (54%)
	15,000-30,000 Rs/-	174 (45%)
	31,000-50,000 Rs/-	2 (0.5%)
	>50.000 Rs/-	2 (0.5%)
Age of the Mother		
5	<20 Years	7 (1.8%)
	20-34 Years	360 (93.5%)
	35 Years and Above	18 (4.7%)

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Marital Status		
6	Married	360 (94%)
	Separated	6 (1.6%)
	Widow/Divorced	19 (4.9%)
Occupation		
7	Housewife	183 (48%)
	Working	202 (53%)
Education		
8	Illiterate	293 (76%)
	Primary	77 (20%)
	Secondary	13 (3.4%)
	Higher	2 (0.5%)
Under 5 Year Children		
9	0-2	242 (62.9%)
	3-5	137 (35.6%)
	>5	6 (1.6%)
Total no. of Children		
10	1-3	213 (55.3%)
	4-6	152 (39.5%)
	>6	20 (5.2%)
Age of Mother at the Time of 1st Birth		
11	17-20 Years	226 (58.7%)
	21-24 Years	135 (35.1%)
	25 Years and Above	24 (6.2%)

In current study, child carrying practices of the mothers were also assessed. It was found that nearly 65% (250) of the children in study area were breastfed while 31% (121) were immediately breastfed after birth. Majority of the children were not given any kind of pre-lactation food (n= 278, 72%). Dietary practices of under 2-year children were analysed and it was found that only 31% (n= 120) of the children were bottle fed during last 24 hours. While only 53% (n= 205) of the children consumed fruits and vegetables during last 24 hours. A brief summary is given in table 2.

Table 2: Child Carrying and Dietary Practices

S. No.	Variables	Frequency (%)
Was this Child ever Breastfed?		
1	No	135 (35%)
	Yes	250 (65%)
How many Hours after the Birth was the Child Breastfed?		
2	Immediately	121 (31%)
	Between 1-24 hours	157 (41%)
	After a day	107 (27%)
Is the Child Still Breastfeeding?		
3	No	116 (30.1%)
	Yes	269 (69.9%)
What type of Pre-Lactation Food/Fluid the Child wasProvided with?		
4	None	278 (72.2%)
	Water	28 (7.3%)
	Butter/Honey	41 (10.6%)
	Cow/Camel/Goat milk	38 (9.9%)
Period of Exclusive Breastfeeding		
5	<4 months	114 (29%)
	4-6 months	124 (32%)
	>6 months	147 (38%)
Reasons for not Breastfeeding		
6	Maternal health issues	27 (7%)
	Refusal of child	21 (5.5%)
	Maternal pregnancy	36 (9.4%)
	None	301 (78.2%)
Weaning Status of the Child		
7	None	185 (48.1%)
	Partial	116 (30.1%)
	Full	84 (21.8%)
Age of Child when Breastfeeding Stopped		
8	0-6 months	267 (69.4%)
	7-14 month	18 (4.7%)
	>14 months	100 (26%)
Age of Child at the Time of Complementary Feeding		
9	0-6 months	295 (76.6%)
	7-14 month	86 (22.3%)
	>14 months	4 (1%)
How many Times was the Child Fed Solid Food during Last 24 Hours?		
10	<3 times a day	209 (54.3%)
	>3 times a day	176 (45.7%)
Was the Child Bottle Fed during the Last 24 Hours?		
11	No	265 (68.8%)
	Yes	120 (31.2%)
What is the Method of Feeding the Child has been Used?		
12	Bottle	70 (19%)
	Cup	63 (16.4%)
	Spoon	30 (7.8%)
	None	219 (56.9%)
Did the Child use Salt during the Last 24 Hours?		
13	No	48 (12.5%)
	Yes	337 (87.5%)
Did the Child take Fruits/Vegetables during the Last 24 Hours?		
14	No	180 (47%)
	Yes	205 (53%)
Did the Child take Egg/Meat during the Last 24 Hours?		
15	No	219 (56.9%)
	Yes	166 (43.1%)

Based on anthropometric assessment, height for age, the findings of this study revealed that 95.9% (n= 369) of the participant children were stunted with a z-score below -2 S.D of WHO standards. Out of these, 30 children (7.8%) were severely stunted (Figure 1).

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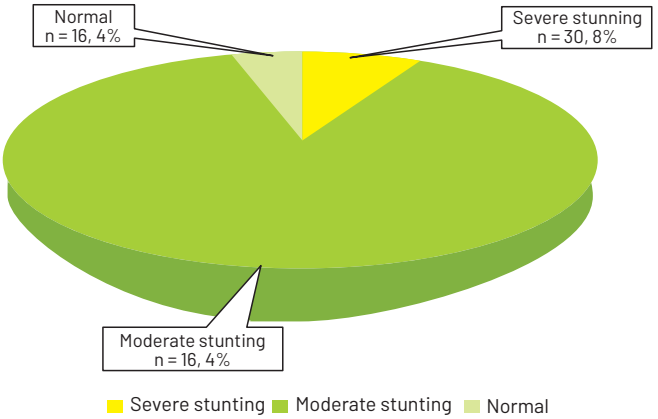

Figure 1: Proportion of Stunting among Under 2-Year Children

The association of stunting with socio-demographic variables, child-carrying characteristics of the mothers, and dietary patterns of the children was determined using the Pearson Chi-Square Test of Independence after confirming the assumptions of the test. It was observed that age of the child, birth order of the child, family income, and total number of children in the household were significantly associated with stunting among under 2-year children (p-value <0.05). Furthermore, children who were breastfed were less prone to stunting as compared to those who were never breastfed (p value<0.05). Similarly, children who were immediately breastfed after birth were less prone to stunting as compared to children who were breastfed after 1-24 hours or 1 day (p value<0.05). Moreover, children who were exclusively breastfed for more than 6 months were less prone to stunting as compared to those with less duration of exclusive breastfeeding (p value<0.05). The age at which breastfeeding stopped and consumption of fruits and vegetables were also found significantly associated with stunting (p value<0.05). A summary of these results is given in table 3.

Table 3: Association of Socio-demographic characters, child carrying characteristics of the mothers, and dietary patterns of the children with Stunting under 2 years' children

S. No.	Variables	Stunting		X^2 (df)	p-Value
		Stunted Frequency (%)	Normal Frequency (%)		
1	**Gender**			0.04 (1)	0.840
	Male	175 (46%)	8 (2%)		
	Female	194 (50%)	8 (2%)		
2	**Age of Child**			23.25 (2)	0.000
	6-10 months	88 (22.9%)	0 (0%)		
	11-17 months	170 (44.2%)	2 (0.5%)		
	18-23 months	111 (28.8%)	14 (3.6%)		
3	**Birth Order of the Child**			7.26 (2)	0.026
	1-3	272 (70.6%)	7 (1.8%)		
	4-7	95 (24.7%)	9 (2.3%)		
	>7	2 (0.5%)	0 (0%)		
4	**Livestock**			2.34 (1)	0.126
	No	210 (55%)	6 (2%)		
	Yes	159 (41%)	10 (3%)		
5	**Family Income**			12.84 (3)	0.005
	<15,000 Rs/-	196 (50.9%)	11 (2.9%)		
	15,000-30,000 Rs/-	170 (44.2%)	4 (1%)		
	31,000-50,000 Rs/-	2 (0.5%)	0 (0%)		
	>50,000 Rs/-	1 (0.3%)	1 (0.3%)		
6	**Age of Mother**			0.39 (2)	0.82
	<20 years	7 (1.8%)	0 (0%)		
	20-34 years	345 (89.6%)	15 (3.9%)		
	35 years and above	17 (4.4%)	1 (0.3%)		
7	**Occupation of Mothers**			0.75 (1)	0.386
	Housewife	318 (83%)	15 (4%)		
	Working	51 (13%)	1 (0.3%)		
8	**Education of Mother**			7.67 (3)	0.053
	Illiterate	278 (72%)	15 (3.9%)		
	Primary	77 (20%)	0 (0%)		
	Secondary	12 (3%)	1 (0.3%)		
	Higher	2 (0.5%)	0 (0%)		
9	**Age of mother at the Time of 1st Birth**			4.85 (2)	0.08
	17-20 years	219 (56.9%)	7 (1.8%)		
	21-24 years	129 (33.5%)	6 (1.6%)		
	25 years and above	21 (5.5%)	3 (0.8%)		
10	**No. of under 5-Year Children**			0.27 (2)	0.87
	0-2	232 (60.3%)	10 (2.6%)		
	3-5	131 (34%)	6 (1.6%)		
	>5	6 (1.6%)	0 (0%)		
11	**Total no. of Children**			8.97 (2)	0.01
	1-3	29 (7.5%)	4 (1%)		
	4-6	140 (36.4%)	12 (3.1%)		
	>6	20 (5.2%)	0 (0%)		
12	**Was this Child Ever Breastfed?**			11.33 (1)	0.001
	No	115 (29%)	20 (5%)		
	Yes	100 (25%)	150 (39%)		
13	**How many hours after the Birth was the Child Breastfed?**			14.0 (2)	0.001
	Immediately	40 (10%)	81 (21%)		
	After 1-24 hours	92 (24%)	65 (16%)		
	After a day	66 (17%)	41 (10%)		
14	**What type of Pre-Lactation Food/Fluid the Child was Provided with?**			2.36 (3)	0.50
	None	269 (70%)	9 (2.3%)		
	Water	26 (7%)	2 (0.5%)		
	Honey/Butter	38 (10%)	3 (0.8%)		
	Cow/Camel/Goat milk	36 (9.4%)	2 (0.5%)		
15	**Period of Exclusive Breastfeeding**			10.34 (2)	0.01
	<4 months	60 (15%)	54 (14%)		
	4-6 months	50 (12%)	74 (19%)		
	>6 months	60 (15%)	87 (23%)		
16	**Weaning Status of the Child**			5.57 (2)	0.61
	None	181 (48%)	4 (1.1%)		
	Partial	107 (28%)	9 (2.4%)		
	Full	76 (20%)	3 (0.8%)		

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17	**Age of Child when Breastfeeding Stopped**			8.20 (2)	0.02
	0-6 months	261 (67.8%)	6 (1.6%)		
	7-14 months	27 (7%)	2 (0.5%)		
	>14 months	81 (21%)	8 (2.1%)		
18	**Age of the Child at the Time of Complementary Feeding**			4.46 (2)	0.11
	0-6 months	283 (73.5%)	12 (3.1%)		
	7-14 months	83 (21.6%)	3 (0.8%)		
	>14 months	3 (0.8%)	1 (0.3%)		
19	**How many Times was the Child Fed Solid Food during the Last 24 Hours?**			2.88 (1)	0.08
	<3 times	197 (51.2%)	12 (3.1%)		
	>3 times	172 (44.7%)	4 (1%)		
20	**Was the Child Bottle-Fed during the Last 24 Hours?**			2.71 (1)	0.10
	No	251 (65%)	14 (3.6%)		
	Yes	118 (30.6%)	2 (0.5%)		
21	**Did the Child use Salt during the Last 24 Hours?**			2.37 (1)	0.12
	No	48 (12.5%)	0 (0%)		
	Yes	321 (83.4%)	16 (4.2%)		
22	**Did the child take fruits/vegetables during the last 24 hours?**			4.37 (1)	0.036
	No	81 (22%)	99 (25%)		
	Yes	79 (20%)	126 (33%)		
23	**Did the Child take Meat/Eggs during the Last 24 Hours?**			0.322 (1)	0.57
	No	211 (54.8%)	8 (2.1%)		
	Yes	158 (41%)	8 (2.1%)		
24	**Is the Child Still Breastfeeding?**			8.30 (1)	0.43
	No	106 (27.5%)	10 (2.6%)		
	Yes	263 (68.3%)	6 (1.6%)		
25	**Complementary Food in the Last 48 Hours in Addition to Breastfeeding**			28.46 (1)	0.17
	No	40 (10.4%)	9 (2.3%)		
	Yes	329 (85.5%)	7 (1.8%)		

DISCUSSION

Current study was carried out at urban tehsil Umerkot, Pakistan. The key goal of the study was to determine the level of stunting and its associated issues among children under 2 years in study area. Study results revealed that nearly 96% (n= 360) of the children out of total 385, were stunted out of which 8% (n= 30) were severely stunted. This is quite an alarming number which highlights the need of nutritional interventions and routine nutritional assessments in such remote areas of the country. Current study percentage of stunting is quite high as compared to previous studies conducted in different areas of Pakistan. A study conducted in Lahore, Pakistan reported the prevalence of stunting among male children to be around 67% while in female children, it was reported to be 33% [13]. Similarly, another study conducted in 2019 found the overall prevalence of stunting among under 5 years' children to be around 44% [6]. The high percentage of stunting in current study population could be due to demographic characteristics of the area as this study was conducted in a remote area of Pakistan where level of education is low, people are mostly illiterate. Therefore, their nutritional knowledge is also quite unsatisfactory which ultimately affects the feeding practices of the children and lead them to nutritional deficiencies like stunting. Results of the current study revealed that age of the children was significantly associated with stunting (p value=0.0001). It was noted that out of total 96% (n= 360) stunted children, 44% (n= 170) children were 11-17 months of age. Study conducted in Mexico in 2020 found that nearly 12.3% of children were stunted and their age was approximately 6-35 months [14]. Birth order of the children was also found significantly associated with stunting (p value= 0.026). It was noted that children with first order, second order and third order were more prone to stunting (n= 272, 70.6%) as compared to others. These findings are not completely supported by the previous literature. A study that was conducted in 2018 found that children in the first birth order have a lower risk for stunting (20%, p-value<0.01). The income level of the family was also found statistically significant with stunting among under 2 years' children in the current study (p value= 0.005). It was observed that children belonging to families with income less than 15000 Rs/- were more likely to be affected by stunting (n= 196, 50%). These results were somehow consistent with the previous literature. A study that was conducted in Indonesia in 2020, found that family income was positively associated with stunting among children aged 0.5-12 years [15]. Breastfeeding was also found to be a significant factor that can affect stunting among under 2 years children (p value= 0.001). It was noted that out of the total study population, 135 children (35%), and 115 children (29%) were stunted. This showed that breastfeeding can be an important factor in preventing stunting among children. These results are consistent with previous literature. A study conducted in Indonesia in 2019 also reported that stunting decreased with exclusive breastfeeding (p value=0.042) [16]. In the current study, it was also noted that out of the total population children who were breastfed immediately after their birth, were the least stunted (n= 40, 10%) as compared to other groups. These findings are statistically significant (p value=0.001) and consistent with the prior literature available [16]. Current results showed that the period of breastfeeding also contributed significantly towards stunting (p value=0.006). It was found that children who were breastfed exclusively for less than 4 months were more stunted (n= 60, 15%) as compared to other groups. These findings can be

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supported by previous literature available. A study that was carried out in Indonesia in 2020, found that breastfeeding can prevent stunting, and the period of exclusive breastfeeding is an important factor for predicting stunting among under 5 years children (p value=0.039) [17]. In the present study, it was noticed that the children who stopped breastfeeding before the age of 6 months or at the age of 6 months, were more likely to be stunted (n= 261, 68%) as compared to other children who breastfed for longer duration and these results were statistically significant (p value=0.01). These findings are contrary to the previous literature. A study conducted in Ethiopia in 2015 revealed that children who breastfed for more than 24 months were prone to stunting [18]. The current study revealed that stunting was less reported among children who consumed fruits and vegetables (n= 79, 20%) as compared to those who did not consume fruits and vegetables (n= 81, 22%) and these findings were statistically significant (p value=0.036). A study conducted in 2015 in Brazile, found that nutritional deficiencies were higher among those children who did not consume fruits and vegetables [19, 20]. So, the current results are similar to the previous findings.

CONCLUSIONS

It was found that the majority of the children under 2 years of age were stunted in the study area. Various factors can play an important role in preventing stunting. Results of the study revealed that breastfeeding and duration of exclusive breastfeeding are important in preventing stunting.

Authors Contribution

Conceptualization: US
Methodology: MS
Formal analysis: NUS
Writing, review and editing: SFK, US

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

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Melamine a Potent Carcinogen: Detection in Packed and Unpacked Milk Samples Collected from Different Regions of Lahore, Pakistan by ELISA Method

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ABSTRACT

Melamine is a nitrogen-rich compound often added to milk to enhance its protein contents. The European Commission has established a permissible limit of 2.5mg/kg for melamine, as excessive amounts can lead to kidney stones and other health risks for both infants and adults. **Objective:** To assess melamine concentrations in both packed and unpacked milk samples from various regions in Lahore, Pakistan. **Methods:** An experimental study was conducted using the Enzyme-Linked Immunosorbent Assay (ELISA) technique to detect melamine levels in milk samples. Measurements were obtained at a wavelength of 450 nm, following standard ELISA protocols. **Results:** The analysis revealed that one unpackaged milk sample contained melamine levels exceeding the permissible limit at 6.3+0.1mg/kg, while the other samples were deemed safe for consumption. Additionally, while melamine was detected in packed milk samples, all were within safe consumption levels. **Conclusions:** In conclusion, melamine was not found in the packaged milk samples, adhering to the acceptable limits set by the European Commission in 2009; however, one unpackaged milk sample showed a concerning level of melamine.

INTRODUCTION

Milk is very nutritious for people and serves as an energy source, supplying lactose, fats, proteins, calcium, vitamins, and minerals [1]. Adulteration refers to the deliberate addition, replacement with lower-quality stuff, or elimination of precious elements from food for personal gain, whereas food fraud encompasses the purposeful inclusion of harmful ingredients [2]. This study presented a colorimetric method for detecting melamine in milk using gold nanoparticle aggregation, offering a rapid and visual detection approach [3]. In most regions of Pakistan, low-quality milk is available, and individuals struggle to find nutritious foods. Several adulterants find their way into milk, such as detergents, water, cane sugar, rice flour, starch, formalin, hydrogen peroxide, sodium chloride, melamine, urea, cyanuric acid, vegetable oil, ammonium sulfate, caustic soda, boric acid, glucose, sorbitol, hypochlorite, salicylic acid and arrowroot [4]. Melamine is an organic industrial substance rich in nitrogen. The compound is NC-NH2, a trimer of cyanimide consisting of 67% nitrogen. C3H6N6 is the chemical composition of the

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compound. This systematic review and meta-analysis evaluates the levels, detection methods, and health risks of melamine and cyanuric acid in milk [5, 6]. Araujo et al., 2012 state that hydrolyzed melamine decomposes into ammelide, ammeline, and cyanuric acid [7]. It is commonly utilized in the manufacture of melamine resins through the reaction with formaldehyde [8]. Additionally, melamine is utilized in the production of plastics, decorative laminates, tableware, molding materials, coatings, flame retardant agents, and adhesives [9]. Besides its various industrial applications, melamine has seen growing utilization in food and dairy items as a synthetic protein source [10]. Due to its elevated nitrogen levels (66%), it is unlawfully included in baby formulas, animal feed, nitrogen fertilizers and milk products as an enhancer of protein [11]. The addition of melamine to packaged milk has become a widespread practice, as businesses employ it to boost market prices, enhance nitrogen levels in milk, and lower product expenses [12]. While melamine is safe in minimal amounts, a TDI (tolerable daily intake) threshold of 0.2 mg/kg has been established using dose-response evaluations from sub-chronic rat research. Additionally, safety thresholds of 1mg/kg melamine in baby formulas and 2.5mg/kg in other products have been set. Many countries have established Maximum Residue Limits (MRL) for melamine across various products to protect public health and ensure food safety. For example, the USFDA established the MRL of melamine in dairy products, milk foods and milk at 0.25 mg/kg, stressing that infant formula available to consumers of United States must be completely melamine-free, whereas the European Union (EU) set the MRL of melamine in dairy items and high-protein foods at 2.5 mg/kg [13, 14]. The present research can aid this effort in Pakistan by concentrating on the easy determination of melamine levels in milk products, marking an initial step toward regulating melamine in food items from local markets. Thus, the primary objectives of this investigation aimed to measure melamine levels and its associations between the protein concentrations of different packaging samples of milk and milk baby formulae with a foundation of local marketplaces in Pakistan.

To accomplish these goals, ELISA based approach was employed to evaluate the presence of melamine in different milk items.

METHODS

This experimental study employed the AgraQuant@ Melamine ELISA kit was used for the current experimental study. It contained melamine standard solutions (0, 20, 100 and 1000ppb), melamine enzyme conjugate, substrate solution, stop solution and wash buffer. ELISA microplate reader (Model: EZ Read 2000, biochrom), centrifuge machine, digital pipettes and microtubes, distilled water were also required for the test. Various samples of packed and unpacked milk (n=10 for packed and n=10 for unpacked) were collected randomly from different locations of Lahore. The unpacked milk samples were obtained from local market and milkmen providing milk at domestic levels in different regions of Lahore. The packed milk samples were collected from some highly recommended and commonly known supermarkets and stores from various locations in Lahore. The samples were kept in refrigerator after collection at -20ºC. 5mL of milk samples were pipetted out in tubes for centrifugation for 10 minutes at 1500rpm. The fat layer was removed and the clear milk serum was collected for analysis. 150µL of the melamine standards and samples were poured into antibody coated wells. 50µL of the conjugate was also added and mixed well by carefully pipetting up and down 3 times. These wells were incubated for 30 minutes at room temperature. The contents of the microwells were emptied and washed with diluted wash buffer solution for a total of 4 washes each. The wells were dried on multilayer absorbent paper towel. 100µL of the substrate solution was added to the wells and then kept for 20 minutes and room temperature. 100µL of the stop solution was added and noted the absorbance within 10 minutes at 450 nm by using an ELISA microplate reader [15]. A standard curve using the absorbance values of the melamine standards was constructed and calculated the melamine concentration in the milk samples based on the standard curve.

RESULTS

LOD and LOQ of the ELISA kit method was 8.0µg/kg and 0.1-0.5mg/kg as prescribed on Kit brochure. Melamine levels were noted in mg/kg (ppm) with a set of 3 readings for each sample and made an average value out of it for each sample. Table 1 shows different readings of melamine observed under ELISA reader and their average value in mg/kg in unpacked milk samples.

Table 1: Melamine Levels (mg/kg) in unpacked Milk Samples

Samples	Value 1 (mg/kg)	Value 2 (mg/kg)	Value 3 (mg/kg)	Average (mg/kg) Mean ± SD
1	6.2	6.4	6.3	6.30 ± 0.10
2	1.29	1.35	1.32	1.32 ± 0.03
3	1.46	1.52	1.49	1.49 ± 0.03
4	0.72	0.78	0.75	0.75 ± 0.03
5	NOT DETECTED			
6	NOT DETECTED			
7	NOT DETECTED			
8	0.56	0.62	0.59	0.59 ± 0.03
9	0.50	0.90	0.70	0.70 ± 0.20
10	0.13	0.19	0.16	0.16 ± 0.03

The results in figure 1 showed that sample 1 is unfit for human consumption as it has melamine beyond permissible levels of 2.5mg/kg.

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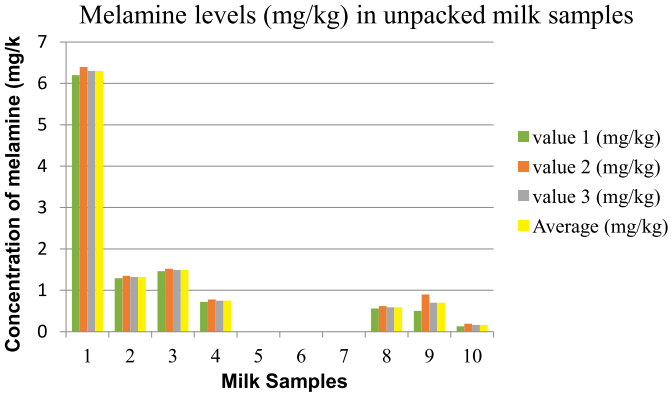

Figure 1: Comparison of melamine levels (mg/kg) in unpacked milk samples

Melamine concentrations detected in unpacked milk samples collected from different regions of Lahore (Table 2).

Table 2: Results of unpacked Milk Samples

Samples	Melamine in unpacked samples (mg/kg)	Results
1	6.30	Not fit for human consumption
2	1.32	Fit for human consumption
3	1.49	Fit for human consumption
4	0.75	Fit for human consumption
5	0	Fit for human consumption
6	0	Fit for human consumption
7	0	Fit for human consumption
8	0.59	Fit for human consumption
9	0.70	Fit for human consumption
10	0.16	Fit for human consumption

Although; the melamine contamination was found in other samples but that was within the safest permissible levels (Figure 2). However, sample 5, 6 and 7 was found with no melamine contamination.

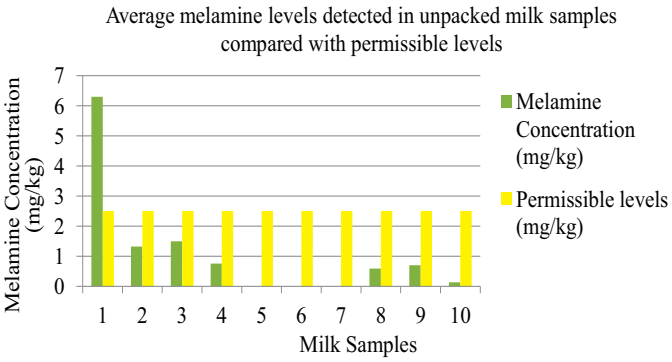

Figure 2: Comparison of unpacked Samples with Permissible Levels

3 readings of each sample were taken by ELISA reader at 450nm and calculated an average value out of it. Table 3 shows different readings of melamine in milk of packed samples. Figure 3 showed melamine levels (mg/kg) in 10 selected samples.

Table 3: Melamine levels (mg/kg) in packed samples

Samples	Value 1 (mg/kg)	Value 2 (mg/kg)	Value 3 (mg/kg)	Average (mg/kg) Mean ± SD
1	0.071	0.063	0.069	0.067 ± 0.004
2	0.168	0.175	0.164	0.169 ± 0.006
3	0.137	0.142	0.148	0.142 ± 0.006
4	0.13	0.127	0.124	0.127 ± 0.003
5	0.114	0.110	0.115	0.113 ± 0.002
6	0.118	0.123	0.122	0.121 ± 0.002
7	NOT DETECTED			
8	0.82	0.86	0.84	0.84 ± 0.020
9	NOT DETECTED			
10	0.37	0.33	0.35	0.35 ± 0.020

The permissible level or the level up to which the consumption of melamine in milk is safe is observed to be 2.5mg/kg by European Commission 2009.

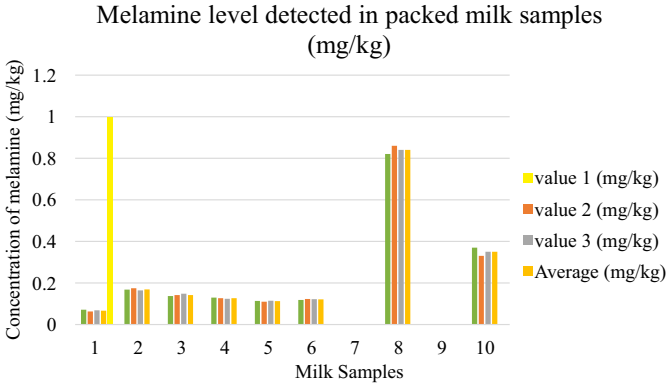

Figure 3: Comparison of Values of Packed Samples in mg/kg

By comparing packed milk samples with permissible levels of EU, 2009, it was noted that all milk samples were safe for human consumption (Table 4).

Table4: Results of Packed Milk Samples

Samples	Average level of Melamine in packed samples (mg/kg)	Results
1	0.067	Fit for human consumption
2	0.169	Fit for human consumption
3	0.142	Fit for human consumption
4	0.127	Fit for human consumption
5	0.113	Fit for human consumption
6	0.121	Fit for human consumption
7	0	Fit for human consumption
8	0.84	Fit for human consumption
9	0	Fit for human consumption
10	0.35	Fit for human consumption

Packed milk has lower levels of melamine as compared to the unpacked milk samples (Figure 4).

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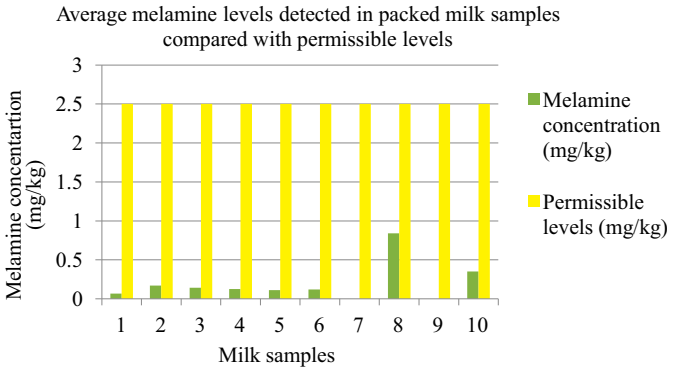

Figure 4: Comparison of packed samples with permissible level

Figure 5 depicted the comparison of melamine concentration in unpacked and packed milk samples alongwith permissible levels.

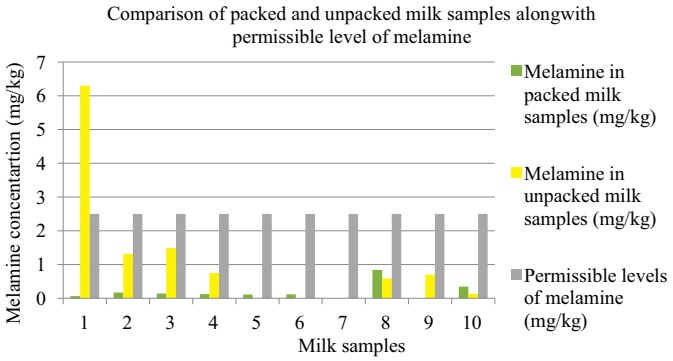

Figure 5: Comparison of Packed and Unpacked Milk Samples

DISCUSSION

An article reviewed advancements in melamine detection, highlighting the evolution from traditional methods to innovative electrochemical sensor technologies [17]. Another study presented a rapid immunochromatographic assay using luminescent quantum dot beads for sensitive melamine detection in milk. [18]. In a study, 300 samples of milk and dairy products that were bought from well-known Turkish merchants were analyzed using the technique. While 2% of cheese, 8% of milk powder, and 44% of yogurt samples had melamine at levels of 121, 694 ± 146, and 294 ± 98 µg/kg, respectively, infant formulae and pasteurized UHT milk did not contain melamine. These results fell short of the thresholds established by EU law and the Codex Alimentarius Commission [18]. These results showed that packed samples were within permissible levels established by the Codex Alimentarius Commission (2010) and EU regulations (European Commission, 2002, 2009; 1 mg/kg for infant formula, 2.5 mg/kg for dairy goods)[19]. In a study conducted by Gouri, 2025, five distinct brands of protein powders underwent FTIR analysis, resulting in spectra for each sample. Eighty percent of the samples indicated the presence of melamine. To prevent melamine contamination and safeguard consumer safety, stringent quality control protocols and routine testing are recommended [20]. The contamination of one unpacked sample found beyond permissible levels is risky and smaller quantities found were due to contamination during the processing of dairy items, while the larger quantities were due to intentional inclusion. Eating foods with these minimal amounts of melamine poses no health risk to consumers. Despite progress, obstacles remain, such as the intricate and variable nature of milk composition, the significant expenses associated with advanced technology, the requirement for specialized knowledge, and the absence of uniform protocols [21].

CONCLUSIONS

Melamine was absent in packaged milk samples and within the acceptable limits set by the European Commission in 2009; however, one sample of unpackaged milk revealed a significant presence of melamine. Additionally, some unpackaged milk samples showed minimal or no detectable levels of melamine. Therefore, this survey indicates that consumers of unpackaged milk face a certain risk of melamine exposure, while packaged milk presents a lower or negligible risk.

Authors Contribution

Conceptualization: NZ
Methodology: MKS
Formal analysis: AJ, MS, SN
Writing, review and editing: NZ, AS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

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Whole Orange Powder as A Rich Source of Polyphenols, Flavonoids and Antioxidants

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ABSTRACT

On a global scale, consumers' faith in dietary therapy for illness remediation has been bolstered by health claims for phytochemical-containing foods, including fruits and vegetables. Thanks to their antioxidant capabilities and chemical variety, polyphenols gave dietary supplements and nutraceuticals new life. **Objectives:** To investigate the antioxidant activity and phytochemicals (TPC and TFC) of the entire orange powder while extracted in water, ethanol and methanol. **Methods:** Each extract was tested for its total flavonoid composition using the aluminium chloride technique and its total polyphenolic content using the Folin reagent. The DPPH test was used to measure the antioxidant activity. **Results:** Results demonstrated that whole orange powder water extract had the lowest total phenolic content values (167.2 ± 3.3 mg GAE/g), flavonoids (35.8 ± 0.2 mg QE/g) whereas methanol extracts displayed the highest values (350.8 ± 6.3mg GAE/g; 72.5 ± 2.2 mg QE/g) and ethanol extracts showed the moderate values (283.4 ± 5.2 mg GAE/g; 57.4 ± 1.8mg QE/g) respectively. At a concentration of 20-100µg/ml, the methanol extract had the greatest antioxidant% inhibition value, 38.50 ± 1.3-87.67 ± 2.4%, followed by the ethanol extract (28.70 ± 1.1-65.40 ± 2.1%) whereas the water extract had the lowest antioxidant% inhibition value, 17.95±0.3-52.25 ± 1.6% and it showed a statistically significant difference values ($p<0.05$) among the extracts. **Conclusions:** It was concluded that the antioxidant levels, polyphenols and flavonoids in whole orange powder were strongly affected by the solvent type employed for extraction, with methanol being the solvent of choice.

INTRODUCTION

Every year, more than 100 million tons of citrus fruits are produced throughout the world, marking a remarkable increase in output. The fruit juice extraction process is the lifeblood of this booming sector, but it also produces copious amounts of waste items, including seeds, pulp, and peels. Half of the fruit is not fit for human consumption, leading to an annual waste of 60 million tons [1, 2]. The peels of citrus fruits, which make up more than half of the fruit itself, are often wasted and ignored, which has serious consequences for the environment, the economy, and our nutrition. These problems are made worse in emerging nations like India due to their inadequate infrastructure for managing this massive amount of biomass. To encourage environmentally friendly production and consumption, the European Union and the United Nations have made efforts in the last decade to redistribute and valorize food waste under a hierarchical system. One of the biggest producers of citrus (Oranges),

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which is aptly referred to as the king of all simple peeler kinds and surpasses the greatest varieties worldwide, is Pakistan. Furthermore, in Pakistan's overall fruit culture, citrus is the country's main crop in terms of both area and production. With a production level of 2.29 million tons, these fruits are cultivated on 210.47 thousand hectares in the Punjab province [3]. There is a lost chance to extract useful bioactive chemicals from citrus peels and entire trash oranges, which are now thrown away. The wide variety of chemicals that make up these compounds has made them famous for the positive effects they may have on health [4]. Citrus peel extracts have not been well investigated. There are several nutritional advantages of eating citrus fruits, which are common in the subcontinental diet. For example, oranges are a good source of flavonoids like narirutin 4'-glucoside, eriocitrin, narirutin, a compound called isosakuranetin, sinensetin, quercetagetin/gossypetin, a medication called no, 3,5,6,7,8,3',4'-heptamethoxyflavone, tangeritin/5-hydroxy-3,7,8,3',4'-pentamethoxy-flavone, Chrysoeriol, limocitrol, limocitrol, and limocitrol, among others, which are most commonly found in orange juice [5]. In addition to a wealth of nutrients, such as vitamins C, A, and B, micronutrients (calcium, phosphorus, potassium), and polyphenols (carotenoids, amino acids, which are triterpenes, phenolic acids, and flavonoids) found in orange peel powder, it is also an outstanding source of dietary fiber [6]. The presence of a group of flavonoids, including narirutin 4'-glucoside (1), eriocitrin (2), narirutin (3), hesperidin (4), isosakuranetin (5), sinensetin (6), quercetagetin/gossypetin (7), nobiletin (8), 3,5,6,7,8,3',4'-heptamethoxyflavone (9), tangeritin/5-hydroxy-3,7,8,3',4'-pentamethoxyflavone (10), Chrysoeriol (11), limocitrin (12), limocitrol (13) (Figure 1) [7].

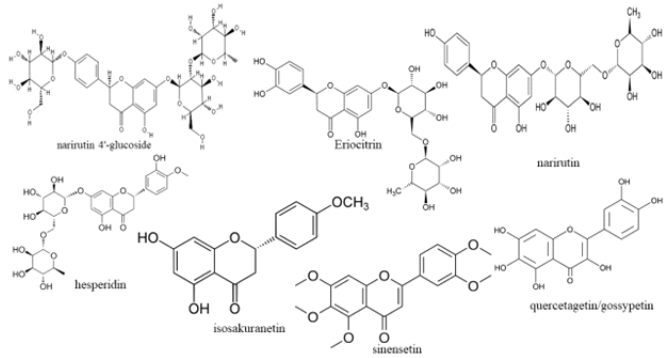

Figure 1: The Presence of a Group of Flavonoids

Using HPLC-DAD, a research by researcher [8] identified nine peaks of polyphenolic compounds, including catechin, caffeic acid, naringin, epicatechin, rutin, quercitrin, quercetin, kaempferol, and luteolin. According to [9], these phenolic compounds can be incorporated into food products or, upon extraction, may be used as natural protectors to shield certain foods from oxidation. Scientific research has shown that orange by-products, which contain a high concentration of bioactive compounds, may have unique and boosted therapeutic effects against oxidative stress in cancer cells, Type 2 diabetes, and cardiovascular diseases [10]. Catechin (peak 1), caffeic acid (peak 2), naringin (peak 3), epicatechin (peak 4), rutin (peak 5), quercitrin (peak 6), quercetin (peak 7) kaempferol (peak 8) and luteolin (peak 9) (Figure 2) [8].

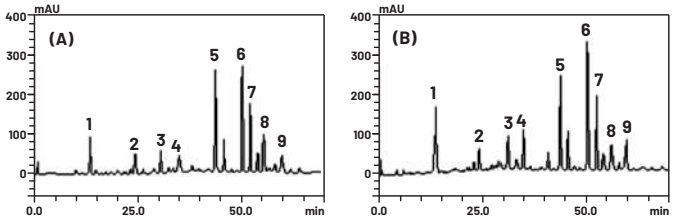

Figure 2: Representative High Performance Liquid Chromatography Profile of Unripe and Ripe Orange Peel Powder

Internally produced reactive oxygen species (ROS) like hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals can cause oxidative stress, which in turn can lead to several diseases, such as cancer, cardiovascular disease, ageing, and neurodegenerative disorders [11, 12]. Exogenous antioxidants from polyphenol-rich foods, such as fresh produce, play a key role in regulating reactive oxygen species (ROS), which in turn has a good effect on human health [13].

This study aims to determine the whole orange powder's polyphenols, flavonoids, its antioxidant activity, and the phytochemical bioactive components. This research has the potential to reveal these byproducts' untapped potential and confirm their status as an affordable, all-natural antioxidant source.

METHODS

After being thoroughly cleaned and chopped, the waste, approximately 40 kg of oranges 500 (Citrus aurantium L.) from the citrus orchard in Sargodha, Pakistan, were dried in a hot air oven dryer for six to eight hours, and the samples were kept in airtight containers [14]. For effective extraction, 20 grams of powdered material was extracted in 200 milliliters of distilled water, methanol, and ethanol, then left at room temperature for 24 hours. After passing the extract through Whatman filter paper No.1, it was kept at 4°C until needed. Following the recommendation of Singleton [15], the total polyphenolic content was determined. To summarize, 2.5 mL of 10% Folin-Ciocalteau's reagent (v/v) was used to oxidize the proper dilutions of the extracts and 2.0 mL of 7.5% sodium carbonate was used to neutralize the reaction. In a spectrophotometer (UV-700 Shimadzu Japan), the intensity of absorption was detected at 765 nm after 40 minutes of incubation at 45°C. After that, the gallic acid

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equivalent was used to determine the total phenol level. As reported earlier, the colorimetric approach was modified to estimate the total flavonoid makeup of both extracts. For this experiment, we mixed half a milliliter of the extract solution with half a milliliter of an ethanol solution containing 20 milligrams per milliliter of $AlCl_3$. The amount of flavonoids was determined as mg QE/g of the extract mixture using the absorbance at 420 nm reading from a wavelength analyzer (UV-1700, Shimadzu Japan) after an hour of incubation at 25°C. The DPPH, which (2,2-diphenyl-1-picrylhydrazyl) scavenging technique, was used to assess the anti-radical activity of the extracts and was slightly modified. A purple DPPH solution (20 mg/L in methanol) was added to the extracts (50 µg/mL at the finish throughout the well), and the mixture was left to incubate at a comfortable temperature for 15 minutes. With the use of an ultraviolet (UV)-visible spectrophotometer (UV-1700, Shimadzu Japan), the DPPH radical's decolonization was seen at 517 nm. The result was reported as DPPH antiradical activity as a percentage compared to the control group that received just DPPH and solvent, without the extracts. The mean ± standard deviation (SD) was used to express the results. Analysis of variance unidirectional (ANOVA) was used to identify significant differences ($p<0.05$), and the Tukey test for multiple comparisons was then used.

RESULTS

Results demonstrated that whole orange water extracts had the lowest total phenolic content values (167.2 ± 3.3 mg GAE/g), flavonoids (35.8 ± 1.2 mg QE/g) whereas methanol extracts displayed the highest values (350.8 ± 6.3mg GAE/g; 72.5 ± 2.2 mg QE/g) and ethanol extracts showed the moderate values (283.4 ± 5.2 mg GAE/g; 57.4 ± 1.8mg QE/g) respectively. At a concentration of 20-100µg/ml, the methanol extract had the greatest antioxidant % inhibition value, 38.50 ± 0.3-87.67 ± 2.4%, followed by the ethanol extract (28.70 ± 1.1-65.40 ± 2.1%), and the water extract had the lowest, 17.95 ± 0.3-52.25 ± 1.6% (Table 1).

Table 1: Total Polyphenols and Flavonoids of Various Extracts of Whole Orange Powder

Extracts	TPC (mg GAE/g)	TFC (mg QE/g)
Methanol (Me-OH)	350.8 ± 6.3	72.5 ± 0.1
Ethanol (Et-OH)	283.4 ± 5.2	57.4 ± 0.4
Water (H_2O)	167.2 ± 3.3	35.8 ± 1.2

Data are represented as mean ± SD

The DPPH radical was used to assess the extracts' free radical scavenging capabilities. At the concentration 20-100 µg/ml, the methanol extract exhibited the highest antioxidant DPPH (% inhibiting) value, ranging from 38.50 ± 1.3-87.67 ± 2.4%, while the ethanol extract came in second with a value ranging from 28.70 ± 1.1-65.40 ± 2.1%. The water extraction procedure of whole dried orange peel had the lowest value, ranging from 17.95 ± 0.3-52.25 ± 1.6%, and it showed statistically significant differences in values ($p<0.05$) among the extracts. The availability of several bioactive components, including the flavonoids that greatly determines the antioxidant potential of whole orange powder peel extracts. Preventing cellular damage and chronic illnesses relies on these chemicals' ability to scavenge free radicals and alleviate oxidative stress. Because these bioactive chemicals have variable distributions and solubility, the antioxidant capabilities of the various plant sections and solvents used in this investigation varied significantly (Figure 3).

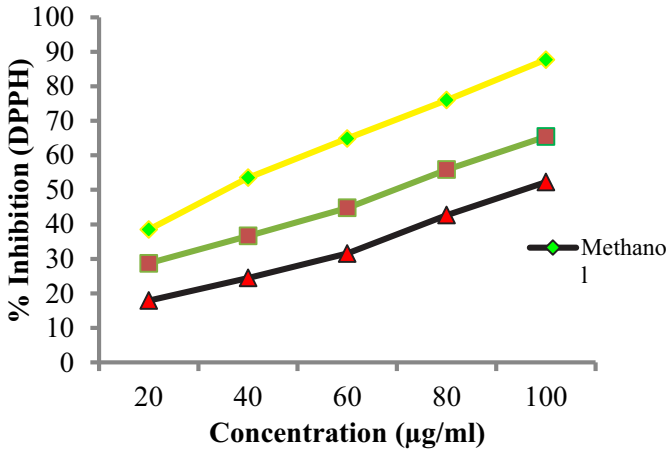

Figure 3: % Inhibition (DPPH) Various Extracts of Whole Orange Peel Powder

DISCUSSION

It was necessary to identify phenolics in food due to the health claims linked with them. The Folin-Ciocalteu technique, which is based on reducing phosphor-tungstic acid to phosphor-tungstic blue, was used for phenolic analysis. This led to a rise in absorbance because there were more aromatic phenolics [16]. If the blue hue becomes more intense, it means that there are more antioxidants in the sample. The orange peel extracts in methanol had the highest concentrations of phenol (350.8 ± 6.3 mg GAE/100 g), and the ethanol extract had TPC (283.4 ± 5.2 mg GAE/g), while the water extract from the whole orange powder showed the lowest concentration of TPC (167.0 ± 3.3 mg GAE/g). The anti-inflammatory, flavor-enhancing, colour-enhancing and phenolic-rich β-carotene found in abundance in orange peels has been linked to the alleviation of several ailments [17]. Carotenoids present in orange peel powder are phytonutrients that are soluble in fat and have effects that prevent cancer, oxidative stress and mutations [18]. The unique citrus scent that has a profound effect on human existence is believed to be mostly produced by flavonoids, which are secondary metabolic product components often present in sweet orange peels [19]. Anticancer,

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antibacterial, antioxidant, anti-inflammatory and anti-allergic are only a few of the many biological effects shown by polyphenols, terpenes and terpenoids [20]. The antioxidant activity of the extracts in this investigation was excellent, with methanol showing the greatest results. Additionally, compared to the ethanol as well as water extracts, the methanol extracts exhibited somewhat greater antioxidant activity. This could be because different solvents extract antioxidant molecules to different degrees. Research has shown that orange peel extracts have strong antioxidant properties; our findings are in line with the literature cited [21].

CONCLUSIONS

It was concluded that whole orange (*Citrus aurantium* L.) powder is a great option due to its high phenolic, flavonoid and antioxidant content. Since they contain bioactive compounds or therapeutic candidates that show considerable action against oxidative stress. It is therefore a good choice for either creating new products or treating or aiding in the therapy of various illnesses. Furthermore, the whole orange powder's antioxidant content was significantly impacted by the kind of extraction solvent used.

Authors Contribution

Conceptualization: MKS
Methodology: MKS, NZ, AS, KS
Formal analysis: SS
Writing review and editing: SN, AAR, SHIA, QUAS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

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Development of an Excel Spreadsheet for Dietary Data Analysis and Calculation of Dietary Inflammatory Index Value for Researchers

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ABSTRACT

The Dietary Inflammatory Index is a measure of the inflammatory load in an individual's diet, based on intake of certain nutrients, food groups, and bioactive compounds. However, computation of the DII is time-consuming and labor-intensive, necessitating precise nutritional information. **Objectives:** To design an Excel spreadsheet for easy calculations of the dietary inflammatory index. **Methods:** Development of the tool was completed in two phases. In phase 1, the Excel spreadsheet was created for nutrients calculation using dietary data, and then using these nutrients for further calculation of dietary inflammatory index, applying a universally referenced dietary inflammatory index, and inflammatory effect scores. In phase 2, validity of the tool was evaluated through different methods, i.e., internal consistency and formula verification, test-retest Reliability, and Statistical Validation. **Results:** The Excel tool was created using the established dietary inflammatory index methodology in seven distinct 7 steps. Face Validity was determined by a three-member panel of expert academic nutritionists who judged the structure, reasonableness, and functionality of the spreadsheet. Content Validity was established by cross-referencing listed nutrient parameters with those that need to be included in peer-reviewed dietary inflammatory index development protocols. The mean difference between dietary inflammatory index scores from the Excel tool and reference manual calculation was near zero (mean bias=0.03), and 95% limits of agreement were -0.21 to +0.27, showing minimal systematic bias. **Conclusions:** It was concluded that the spreadsheet demonstrated strong agreement, high correlation, and statistical reliability, validating it as a practical tool for dietary inflammatory index computation in dietary studies.

INTRODUCTION

Dietary habits influence the body's inflammatory status significantly, which itself plays a central role in the etiology and pathogenesis of most chronic diseases [1]. All such diseases, i.e., cardiovascular disease, type 2 diabetes, metabolic syndrome, and certain cancers, have been linked with chronic low-grade systemic inflammation [2]. Because diet is an important modifiable risk factor, understanding its impact on inflammatory processes is a significant area of research in nutritional epidemiology and public health. Dietary Inflammatory Index (DII) was developed to facilitate the measurement of the inflammatory load of a diet [3]. Since its development, DII has been extensively used and described in many studies [4, 5]. The DII is a publication-based index which imparts scores to food constituents based on their ability to elevate or reduce inflammatory markers such as interleukins, C-reactive protein (CRP), and tumor necrosis factor-alpha (TNF-α). It comprises an extensive range of food parameters (macronutrients, micronutrients, flavonoids, and other bioactive compounds, providing a standardized unit to convey the pro- or anti-inflammatory component of a dietary style [6-10]. While helpful, determination of the DII score could be technically daunting. It requires accurate dietary intake data, standardized nutrient databases, and a knowledge of sophisticated statistical algorithms. This technical limitation tends to limit the use of the DII in small research labs or by researchers who do not have specialized training in nutrition science or database

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administration.

This study aims to develop a spreadsheet-based, user-friendly tool to render the process of DII calculation more accessible. This tool aims to assist researchers, students, and practitioners in conducting proper and effective DII measurements with limited technical expertise. By simplifying data entry and automating computation, our tool is designed to facilitate greater accessibility and promote wider application of DII in diet assessment and chronic disease research.

METHODS

A cross-sectional study employed a development-based and methodological design that was tool-centred. More precisely, this study entailed designing, developing, and validating an Excel tool for nutrient calculation and the Dietary Inflammatory Index (DII) from the nutrients calculated. The details of the methods are given in the following sections. However, briefly, a thorough re-examination of the original DII framework was performed to establish all 45 food parameters, their inflammatory effect scores, and global reference values (means and standard deviations). Data standardization and computational model design were employed. DII calculation formulas (energy adjustment, z-score calculation, centering percentiles, and assigning inflammatory weights) were converted to Excel functions and algorithms. An Excel template was created with in-built formulas, logical functions, and conditional formatting to streamline all six steps of DII calculation (as described in the following section). The Microsoft Excel program was used for the development of the tool. The first sheet of this Excel tool was used for dietary data of the seven food groups. So, for example, in any research study, the dietary intake data collected through 24-hr-Dietary Recall of Food Frequency Questionnaires (FFQ) can be in this sheet. These dietary intake data can be used for the calculation of nutrients. We developed a nutrient calculator on the second sheet of the Excel tool, which provides all nutrients calculated from the dietary intake data on sheet 1. The nutrients on sheet 2 of the Excel tool can be used to calculate DII scores using information from sheet 2 on nutrients. Dietary nutrient calculator (sheet 2) is used for the calculation of nutrients from available diets and mixed dishes. At present, nutrients can be calculated for a total of 120 different foods and mixed dishes of Pakistani origin as reported in numerous studies [11-19]. This sheet can be extended to accommodate more foods and dishes in the future. The details of the development of this calculator can be found elsewhere [20]. Once data on nutrients and other food parameters (a maximum of 45) are available, DII can be calculated using these parameters. For the present study, DII was calculated using the following seven steps. Step 1: Codeletion of Dietary Data: A vast amount of data previously obtained on nutrient intake from our published research [6-25]. The majority of them were obtained using repeated 24-HDRs. Step 2: Nutrients Calculation: Nutrients were computed from the 24-hr-DR data using our in-built home nutrients calculator. The following nutrients were computed: A. Macronutrients: Carbohydrates, fats, and proteins; B. Micronutrients: Vitamins (A, C, E, D, etc.), minerals (magnesium, calcium, etc) C. Bioactive compounds: Polyphenols, flavonoids, and antioxidants; D. Food groups: Fruits, vegetables, meats, and processed foods. Step 3. Calculation of Z-Score: The mean daily intake of each one of the 45 parameters is reported relative to the default global mean as a z-score. This is realized by taking the global daily mean intake of each parameter away from the meal's corresponding average daily intake and dividing the result by its standard deviation (i.e., the global daily mean intake standard deviation). Step 4: Converting Z-scores to centered-percentiles: First, to reduce the influence of right skewing, the z-score is transformed into a percentile score. Second, to obtain a symmetrical distribution with values ranging around 0 (null) and being restricted between -1 (maximally anti-inflammatory) and 1 (maximally pro-inflammatory), each percentile score is doubled and then 1 is subtracted. Step 5: Multiplying centred-percentiles by parameter-specific overall inflammatory effect scores: The parameter-centred-percentile value of every one of the 45 parameters is then multiplied by its corresponding parameter-specific overall inflammatory effect score to give a parameter-specific DII score. Step 6: Summing parameter-specific DII scores: Each of the 45 parameter-specific DII scores is then added up to give the overall DII score for the meal. Step 7: Excel Spreadsheet Design: The spreadsheet tool's design was anchored on a user-friendly format where users can enter food diet data, compute nutrient consumption, and get a DDI score. The most important parts of the tool are: Data Input Section: Table where users can enter the amount (grams, milliliters, servings) of various foods an individual has eaten. Nutrient Content Database: A pre-defined database of typical foods and their corresponding nutrient content, like the inflammatory or anti-inflammatory action of each nutrient. C. DII Calculation Formula: Formulae based on standard DII calculations involving the food intake data to calculate an overall DDI score. The validity of the Excel-based Dietary Inflammatory Index (DII) calculator and its associated data collection sheet was evaluated through the following methods. Internal Consistency and Formula Verification: All of the computational equations incorporated in the Excel program, specifically those about Z-score computation, centered-percentile conversion, and parameter-dependent DII score calculation, were independently checked by two researchers. Cross-

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validation was conducted with a series of mock dietary data sets, and the DII results were tested for consistency and logical accuracy on repeated entries. Test-Retest Reliability: To evaluate the tool's long-term reliability, the same dataset was entered again by various users (nutrition experts) in two sessions with one week of separation. The DII scores obtained from both sessions were compared and were found to have the same values, demonstrating high reproducibility. Inter-Rater Reliability: Three individual raters applied the tool to enter the same dietary information and calculate the DII scores. The outcomes were identical for all users, indicating that the tool generates consistent outputs irrespective of the operator so long as data entry adheres to the outlined instructions. All these steps in aggregate attest to the technical reliability of the tool in generating consistent and accurate DII values, thus enhancing its application for researchers working with dietary data. For validation of the tool, we followed a two-step process of both content and statistical validation. Validation Data Collection: A sample data set was gathered from 40 participants using a 24-hour dietary recall technique. Their daily consumption of 30 food parameters important for the DII calculation (e.g., energy, protein, fiber, saturated fat, vitamin C, iron, etc.) was documented. These values were entered manually into the Excel tool. Criterion Validity: We contrasted DII scores derived from our Excel tool with those computed via the manual method. Bland–Altman Analysis: A Bland–Altman plot was created to determine the agreement between the two techniques. Inter-Rater Consistency (Reliability Check): Three professional nutritionists each entered the same dietary data set into the tool independently. Intraclass Correlation Coefficient (ICC) between the resultant DII scores was assessed. In the present research, the process of validating the Excel-based DII tool was mainly concerned with face validity and content validity, both of which were objectively evaluated using expert review and structured feedback processes. The following is how each was addressed: Face Validity (objectively assessed): Face validity was assessed using a panel of three nutrition and epidemiology experts who independently analyzed the tool's design, clarity of direction, ease of use, and suitability for use in its intended application. Through their comments, it was guaranteed that the tool seemed to screen what it is intended to quantify, efficient and accurate calculation of DII scores. Content Validity (measured objectively): The tool content (i.e., the parameters from DII included, Z-score and percentile conversion formulae, and the incorporation of inflammatory effect scores) was cross-checked against published literature [3] and subsequent revisions to the DII methodology. Expert reviewers agreed that all salient elements were incorporated and well-organized. Although this tool creation did not entail inferential statistical analysis (e.g., hypothesis testing), computational correctness was maintained through manual checks of calculated DII scores against known values; internal consistency validation within the Excel formula logic and trial runs using simulated dietary data to ensure reproducibility and consistency of outcomes. In future use, we expect additional statistical confirmation through empirical research in which DII values generated from the tool will be measured against clinical or inflammatory biomarkers to permit construct and criterion validity testing.

RESULTS

The spreadsheet developed in this study offers an easy-to-use interface for researchers to compute the DDI values of different diets, formulations and mixed dishes of Pakistani origin. There was no significant difference between the mean DII score calculated by the two methods ($p>0.05$). The mean difference between DII scores from the Excel tool and reference manual calculation was near zero (mean bias=0.03), and 95% limits of agreement were 0.21 ± 0.27, showing minimal systematic bias. Intraclass Correlation Coefficient (ICC) between the resultant DII scores was 0.996 (95% CI: 0.993–0.998), reflecting high consistency and reliability between users. The above validation processes establish that the tool generates reproducible, accurate, and reliable DII values, validating its use for diet data analysis purposes in research applications (Table 1).

Table 1: Validation Between the Manual Calculation and Excel Sheet Result

Methods	Mean ± SD	p-Value
Manual Calculation	1.20 ± 0.80	0.216
Excel Sheet Result	1.17 ± 0.81	

The findings show the spreadsheet demonstrated strong agreement, high correlation, and statistical reliability, validating it as a practical tool for DII computation in dietary studies. The Pearson correlation coefficient between the two sets of DII scores was $r = 0.96$ ($p<0.001$), reflecting a very strong positive correlation and excellent concordance (Table 2).

Table 2: Assessment of Accuracy and Reliability

Statistical Methods	Purposes	Results
Pearson's Correlation (r)	Measures the Strength of Linear Relationship	r=0.96, p<0.001
Intra-class Correlation Coefficient (ICC)	Test Consistency Between Methods	ICC=0.95 (95% CI: 0.91–0.98)
Bland–Altman Plot	Assesses Agreement and Bias	Mean difference=0.03 (limits: -0.14 to 0.20)
Paired t-test	Tests for Significance Mean Difference	p=0.21 (not significant)

To evaluate the accuracy of the constructed Excel

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spreadsheet to determine Dietary Inflammatory Index (DII) scores, we compared it using Bland-Altman plotting with a standard DII calculation program. The outcome showed a near-zero mean difference (bias) that implied there was no systematic bias between the two methods. In addition, most of the points lay within the ± 1.96 standard deviation limit of agreement, indicating strong agreement and consistency between the reference method and the spreadsheet. This indicates statistical reliability and validity of the Excel-based tool for correct DII calculation in diet studies (Figure 1).

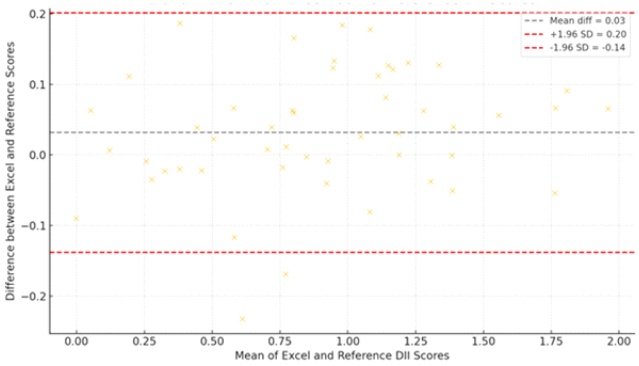

Figure 1: Bland-Altman Plot: Excel tool vs. Manually Calculated DII Score

DISCUSSION

The Dietary Inflammatory Index (DII) is a scientifically confirmed measure to estimate the inflammatory capacity of a person's diet [1]. It examines the consumption of certain nutrients, food groups, and bioactive food components known to induce or reduce inflammation within the body. In this study, we developed an Excel-based tool for two purposes: 1) the calculation of nutrients from food data and 2) the calculation of the DII score from this nutrient database. After the spreadsheet had been created, we carried out a validation procedure by using the tool to test it against several different dietary profiles. Content validity, Test-Retest Reliability, Criterion Validity, Bland–Bland-Altman analysis, and Inter-Rater Consistency (Reliability Check) were employed to assess the overall quality of the tool. All these tests are commonly used for testing new or modified tools [22, 23]. Face Validity: Face validity was attained by showing the Excel spreadsheet to a group of subject-matter experts in nutrition and dietetics. They validated that the format, vocabulary, and structure of the tool were valid, sensible, and in harmony with common dietary assessment practices. Their endorsement confirmed that the tool seems to capture what it is supposed to dietary aspects of inflammatory potential. Content Validity: Content validity was maintained by having registered dietitians and academic researchers perform a thorough review of the included dietary parameters. The food parameters were cross-checked with published literature and matched the validated components employed in the original DII algorithm [3]. The panel ensured that the tool fully captures all pertinent pro- and anti-inflammatory dietary components, including macronutrients, micronutrients, and particular food bioactives. Construct Validity: Construct validity was tested by comparing spreadsheet-generated DII scores with anticipated dietary patterns. For instance, vegetable-, fruit-, and whole-grain-rich diets produced more anti-inflammatory (negative) DII scores, whereas saturated fat- and processed food-rich diets produced more pro-inflammatory (positive) DII scores. This was in line with theoretical predictions and validates the construct validity of the instrument. Accuracy Validation of the Excel-Based DII Calculator: To assist the accuracy of the tool, an accuracy step was performed with the use of parallel dietary data that were computed manually using the original DII computation method [3] and using the Excel spreadsheet that was developed. Both methods' resulting DII scores were compared. Pearson correlation coefficient (r) between the two pairs of DII values was computed and was r=0.96, p<0.001, denoting an extremely strong positive correlation. The Bland-Altman plot also exhibited strong agreement between the methods, with 95% of differences within good limits of agreement. Paired sample t-test findings revealed no statistically significant difference between the spreadsheet DII and manual DII values (p>0.05), in favor of measurement equivalence. These findings validate that the spreadsheet produces reproducible and accurate results, consistent with the control methodology. Accuracy can be improved in future studies by validating the tool using varied population datasets and biomarker correlations (e.g., CRP levels). To evaluate the accuracy of the constructed Excel spreadsheet to determine DII scores, we compared it using Bland-Altman plotting with a standard DII calculation program (manual calculation as a reference). The outcome showed a near-zero mean difference (bias) that implied there was no systematic bias between the two methods. In addition, most of the points lay within the ±1.96 standard deviation limits of agreement, indicating strong agreement and consistency between the reference method and the spreadsheet. This indicates the statistical reliability and validity of the Excel-based tool for correcting DII calculations in diet studies. Bland-Altman is a standard method of comparing two scores obtained with different methods [23]. The tool's format, vocabulary, and structure were valid, sensible, and in harmony with common dietary assessment practices as assessed by experts through face validity assessment, another tool-quality assessment

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method [24]. The content validity, another quality assessment parameter for tools [25], confirmed that the tool fully captures all pertinent pro- and anti-inflammatory dietary components, including macronutrients, micronutrients, and particular food bio-actives.

CONCLUSIONS

It was concluded that the newly developed Excel-based tool is a useful tool for the calculation of nutrients and DII which demonstrated strong agreement, high correlation, and statistical reliability, validating it as a practical tool for DII computation in dietary studies.

Authors Contribution

Conceptualization: IA, FZ, HUR
Methodology: HUR, IA
Formal analysis: HUR, IA
Writing review and editing: HUR, IA, FZ

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Evaluation of Nutritional Knowledge and Lifestyle Habits of Orphan Children Residing in Different Institutes of Peshawar City

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ABSTRACT

The nutritional status of orphaned children residing in Peshawar orphanages is scantily documented. **Objectives:** To assess the eating habits, awareness of nutrition, and dietary status of children living in orphanages in the Peshawar area of Pakistan. **Methods:** The study involved 120 children, ages 5 to 15, and was conducted at three separate orphanages in Peshawar city. Food frequency questionnaires were used to collect data on the orphan children's socioeconomic situation, anthropometric measures (weight and height), eating patterns, physical activity, and food preferences. Additionally, data on biochemical tests used to measure hemoglobin levels (Hb) were gathered. To determine the orphans' knowledge, attitudes, and behaviours about dietary guidelines, another Knowledge, Attitude, and Practice (KAP) questionnaire was used. SPSS-26 was used to analyze the data that were collected. **Results:** According to the findings, 4%, 9%, and 3% of the children in the study were underweight, overweight, and obese, respectively. Additionally, 12% of the orphans were anemic, and 13% were stunted. Carbohydrates, fruits, vegetables, meat, legumes, dairy products, nuts, and beverages were consumed by the majority of the orphans. However, they also have a great preference for chips, pickles, and fast food. **Conclusions:** It was concluded that the current findings indicated poor diet habits and the occurrence of malnutrition among various children's orphanages of Peshawar. The present study reveals the link between malnutrition in orphans and the absence of particular care, which indicates the importance of intervention strategies to diminish the risk of malnutrition for this vulnerable group in the district of Peshawar.

INTRODUCTION

Malnutrition is a frequent public health concern in Pakistan [1-5], and the orphaned children are most affected [6]. Children aged less than 18 years who lost one or both parents are 'orphans'. The number of orphans has risen and is now over 140 million in the world, of which most are residing in Asia and Africa. Orphans are on the rise due to armed conflict, epidemics like HIV/AIDS, and low socioeconomic status. Orphans, particularly in the developing world, suffer from widespread health and nutritional issues, with malnutrition being common [7]. A lot of this vulnerable section of the population has a certain perception regarding healthy diets, and hence, they adopt inappropriate dietary practices and incur nutritional deficiencies [6, 8]. In Pakistan, there are approximately 4 million orphans, mainly due to security issues, natural disasters, and the loss of parents. Orphans in institutions often experience poverty, poor living conditions, poor learning achievements, and increased exposure to abuse and mental illness. Their diet is typically not as good as children raised in families, especially micronutrient deficiency [8]. Similar to the rest of Pakistan, in KP province, although orphans are generally cared for by extended families [9], some of them still lack nutritional information and support systems. Rising costs and shifting values in society are challenging traditional patterns of care. Institutional care in Pakistan does not always meet the emotional and nutritional needs of orphans, potentially increasing the risk of long-term psychological and physical morbidity. The present research specifically focuses on the measurement of major anthropometric parameters

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like height, weight, and body mass index (BMI), as well as on precise body composition measurements like fat mass and lean mass. By relating such measures of physical health with food intake among the children, nutrition awareness, and prevailing feeding patterns in orphanages, the research tries to disentangle determinants influencing their nutritional status. This study aims to examines the impact of caregiving in institutions, the structure of menus, and caregivers' awareness on child development and overall health of these vulnerable children. Through this multi-dimensional approach, the study hopes to offer practical recommendations for policy and practice improvement in child care and nutrition in institutions.

This study aims to comprehensively evaluate the nutritional status, knowledge, attitudes, and feeding practices of 5- to 15-year-old and 15-year-old institutionalized orphan children residing in various orphanages in Peshawar, Khyber Pakhtunkhwa (KP).

METHODS

This study used a cross-sectional design, where data were collected at one-time point [10]. The present research study was conducted in Pakistan's District Peshawar. For the current study, the 5–15-year-old orphans residing in the Peshawar district's lower-level orphan institutions were surveyed. The formula n=N/1 + Ne2 developed by Solvin was used to calculate the sample size. The sample size to be drawn from the population was calculated using this formula. The margin of error and confidence level would be considered using, this calculation. Having a total number of orphan children in the three orphanages (n=200), with a Margin of error e=0.05, the estimated sample required was 133. A master list of potential participants (or schools/institutions) was created first, and all were given a unique identification number. From a computer-generated random number list (Excel), the number of participants required was chosen. To achieve representativeness by institution, stratified random sampling was used, splitting the population into institutions, and randomly selecting samples proportionally from each of these strata from a random number generator. The process listed below was used to choose a random sample: A list of every orphanage in Peshawar was acquired. Using these lists and a random number selection technique, a representative sample was chosen from each orphanage. A total of 120 orphans between the ages of 5 and 15 were chosen. Sociodemographic information, anthropometric measures, 24-hour dietary recall (24 hr-DR) data, and blood hemoglobin levels were among the indicators that were gathered. Anthropometric measures and blood hemoglobin levels were the dependent factors, whereas sociodemographic and food consumption, as assessed by 24-hour DR, were the independent variables. In this study, participants between the ages of 5 and 15 were enrolled as study subjects. Participants with a history of sickness or those who had just recovered from a medical ailment were excluded. The research excluded participants who had recently recovered from any medical issues or who had previously had any sickness. Following BKUC regulations and ethics standards, participant identities were kept anonymous. To examine the data, SPSS version 26 was used. The means and standard deviations of the data were presented. Anthropometric indicators such as weight for age (WAZ), height for age (HAZ), and BMI for age (BAZ) were computed with WHO Anthro Plus software from the WHO Growth Reference for 5–19-year-old children and adolescents (2007) [11]. The Z-scores were obtained according to WHO recommendations for measuring nutritional status and pattern of growth. Before using parametric tests, normality was tested using the Shapiro-Wilk test (for n<50) or the Kolmogorov-Smirnov test (for n ≥50), and visual inspection of Q-Q plots and histograms. Dietary intake values were contrasted with the Recommended Dietary Allowances (RDA) to determine the energy deficit from the RDA and the protein intake deficit from the RDA. RDAS were established according to the age- and sex-related values presented in the Pakistan Dietary Guidelines (2018). For 11–13-year-old participants, the RDA for energy was about 2200 kcal/day for boys, 2000 kcal/day for girls, and for protein was 45–50 g/day, based on sex. These reference values were employed to calculate individual differences between recommended and reported intake. Independent samples t-tests were used only if normality was established. For non-normal continuous data, the Mann-Whitney U test was utilized instead. For categorical variables, Chi-square tests were employed to evaluate associations, and Chi-square assumptions, such as expected cell frequencies ≥5 in at least 80% of cells, were checked. In case of violation of assumptions, Fisher's Exact Test was utilized. A p-value of 0.05 was used as the level of significance.

RESULTS

The general traits of the orphan are displayed in Table 1. The orphans were 12.2 (1.88) years old on average. A significant portion of individuals (14.2%) had both parents deceased. The family size was greater (5-7 individuals) in most situations (75.8%). The average family's monthly income was between 8,000 and 10,000 rupees. The majority of parents were illiterate, according to the parents' educational status (83% of mothers and 83% of fathers) (Table 1).

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Table 1: Overall Student Qualities

Non-Categorical Variables	Mean ± SD	Range
Age	12.23 ± 1.88	5.5 - 15
Weight	38.92 ± 1177	22.0 to 66
BMI	18.38 ±7.25	13.0 to 22
Hb	12.01 ± 1.09	9.8 to 13.5
Categorical Variables		**n (%)**
Parent status	Only Father Dead	113 (94.2%)
	Only Mother Dead	17 (14.2%)
	Both Dead	17 (14.2%)
Family Member	2 to 4	31 (25.8%)
	5 to 7	91 (75.8%)
Monthly Income	8000 to 10000	76 (63.3%)
	1100 to 15000	44 (36.7%)
Father Education	Illiterate	104 (86.7%)
	Middle	11 (9.2%)
	Matric	5
Mother Education	Illiterate	100 (83.3%)
	Middle	10 (8.3%)
	Matric	10 (8.3%)
Physical Activity	Mild	8 (6.7%)
	Moderate	105 (87.5%)
	High	7 (5.8%)
	TV Viewing	80 (66.7%)

The findings show the distribution of the students' characteristics according to their level of nutrition knowledge. According to the findings, pupils who knew a lot about nutrition were taller, heavier, and older. The mean weight (SD) of students with high nutrition knowledge was 38.9 ± 11.31 kg, while that of students with inadequate nutrition knowledge was 38.8 ± 13.55 kg. Both the poor knowledge group (38.8 kg) and the good knowledge group (38.94 kg) had a very similar mean weight. The t-test statistic was t = -0.056 and the p-value = 0.956, i.e., there was no significant difference between the two groups in terms of weight. The mean age poor knowledge group was 11.88 years of age, whereas the good knowledge group was 12.3 years of age. No significant difference was found concerning age (t=-1.492; p-value=0.138). The poor knowledge group had a mean height of 144.1 cm, whereas the good knowledge group's mean height is 146 cm, with no significant difference in height between the two groups (t=-1.236; p-value=0.22). The poor knowledge group has a mean hemoglobin level of 11.85 g/dL, and the good knowledge group has a mean of 12.0 g/dL with no significant difference in hemoglobin level between the groups (t=-0.53; p-value=0.601) The poor knowledge group mean WAZ was -1.16, and that of the good knowledge group was -0.9 with no significant difference in WAZ between the groups (Mann-Whitney U test statistic was U=25.5 and p-value=0.358). The group with poor knowledge had a mean of HAZ=-0.77, and the group with good knowledge had a mean of -0.8 with no significant difference in HAZ between the groups (t=-0.214; p-value=0.832). BAZ scores did not significantly vary between groups (t=0.684, p=0.496). Intake of energy was the same in all groups, and there was no statistically significant difference (t=-0.151, p=0.880). Protein intake did not vary significantly between the two groups (t=-0.958, p=0.340). There was a significant difference in energy intake compared to RDA, with the "Good knowledge" group having a lesser energy deficit than the "Poor knowledge" group (t=-2.795, p=0.006). Likewise, protein intake deviation from RDA was significantly less (more in line with recommendations) in the "Good knowledge" group (t=-3.025, p=0.003) (Table 2).

Table 2: Participants' Distribution by Nutrition Knowledge Level

Variables	Knowledge Score Categories	n	Mean ± SD	SE	Test Statistic	p-Value
Weight	Poor	26	38.8 ± 13.55	2.6575	t=-0.056	0.956
	Good	93	38.9 ± 11.31	1.1733		
Age	Poor	26	11.8 ± 1.84	0.3609	t=-1.492	0.138
	Good	93	12.3 ±1.90	0.1972		
Height	Poor	26	144.1 ± 17.34	3.4015	t=-1.236	0.22
	Good	93	146 ± 17.07	1.77		
Knowledge Score	Poor	26	2.5 ± 1.14	0.2231	t=-13.2	<0.001
	Good	93	5.6 ± 0.87	0.0909		
Attitude Score	Poor	26	3.4 ± 1.17	0.2303	t=-3.98	<0.001
	Good	93	4.5 ± 0.18	0.1228		
Practice Score	Poor	26	10.9 ± 2.54	0.499	t=0.528	0.598
	Good	93	10.3 ± 2.31	0.2386		
Hemoglobin Level	Poor	15	11.8 ± 1.46	0.3786	t=-0.531	0.601
	Good	31	12.0 ± 0.94	0.1689		
WAZ	Poor	7	-1.1 ± 0.92	0.3497	U=25.5	0.358
	Good	12	-0.9 ± 0.74	0.2143		
HAZ	Poor	26	-0.7 ± 1.36	0.2677	t=-0.214	0.832
	Good	93	-0.8 ± 1.85	0.1917		
BAZ	Poor	26	0.06 ± 1.01	1.014	0.1988	0.1988
	Good	93	-0.14 ± 1.54	1.544	0.1601	0.1601
Energy intake (Kcal)	Poor	26	1776.0 ± 528	528.61	103.6689	103.6689
	Good	93	1792 ± 448	448.133	46.4693	46.4693
Protein Intake (g)	Poor	26	35.7 ± 5.53	5.534	1.0853	1.0853
	Good	93	36.9 ± 5.68	5.68	0.589	0.589
Energy Difference from RDA	Poor	26	-219.7 ± 306	306.935	60.1949	60.1949
	Good	92	-52.7 ± 253	253.633	26.4431	26.4431
Protein Intake Difference from RDA	Poor	26	-6.1 ± 6.17	6.168	1.2097	1.2097
	Good	93	-1.2 ± 8.71	8.706	0.9028	0.9028

DISCUSSION

The current study assessed the nutritional status of 120 male orphans, approximately 15 years old, in Peshawar. Findings demonstrated that 16% were normal weight, 9% were overweight, and 3% were obese. Comparisons to research in Nepal, Ethiopia, Lebanon, and Nigeria indicated comparable or slightly different nutritional patterns among

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orphans and vulnerable children [12-15]. Malnutrition rates by categories, such as the Gomez method, the research revealed: 30% had normal, 46% had 1st-degree malnutrition, 25% had 2nd-degree malnutrition, and 2.6% had 3rd-degree malnutrition. The results were similar to the results of similar research on orphaned children in other areas. In this study, 21% of the subjects were anemic, similar to findings from Yemen (19%) [16]. Nonetheless, greater rates were seen in Hodeida (37.8%) (16) and Istanbul (27.6%) [17-18]. In the current study, nutritional knowledge was moderate but not ideal, with most participants having scores slightly above the 50th percentile. There were positive nutrition attitudes, and this is hopeful for future behavioural change. Still, knowledge wasn't sufficient to guarantee healthy practice. Attitude and behaviour change were necessary to improve eating habits. Most children ate fast food and sugary drinks based on taste and convenience. 95% of children took the menu exactly as it came, usually having cereals, pulses, and vegetables. Comparison with studies demonstrated similar patterns of consumption of staple foods but different consumption of green vegetables and dairy [19, 20]. The mean caloric intake (1787 ± 89.6 kcal) fell short of recommended daily allowances (RDAs). Protein as a percentage of total calories was 17–18%, suggesting possible protein-energy malnutrition, particularly in children aged more than 10. Fat and carbohydrate consumption differed according to age but was generally adequate. 14-year-old children had very low energy consumption, covering only 68–71% of RDAs, and this poses a problem for their nutritional status. Findings on diet diversity and risk of malnutrition indicated that orphans who ate from all food groups were at reduced risk of malnutrition. Starchy food- and legume-based diets were prevalent because they are bulky and satiating, but such diets can be lacking in critical micronutrients if not well balanced. Malnourishment is a significant public health problem among institutionalized and orphan children, especially in middle- and low-income nations. Orphan children are at high risk because they have limited access to well-nourished diets, inferior care, and limited health services. This is likely to result in both undernutrition (stunting, wasting, and thinness) and micronutrient deficiency, most notably iron-deficiency anemia. Malnutrition in orphans is often aggravated by unstable food availability, poorly thought-out menus, and lack of nutritional knowledge among caregivers. Irregular supply of food was observed in orphanages where the children were served loads of fruits and calorie-rich foods at some times and only fruits for months at others.

CONCLUSIONS

It was concluded that the general nutritional and health condition of 5–15-year-old orphan children living in institutions all over Peshawar was moderately good as compared to the set standards.

Authors Contribution

Conceptualization: IA
Methodology: AB, IA
Formal analysis: AB, IA
Writing review and editing: AB, IA

All authors have read and agreed to the published version of the manuscript.

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