

# Antioxidant, Chemo-Protective Role Of Buffalo Colostrum And Milk Whey Derived Peptide Against 2, 4-Dinitrophenol Induced-Oxidative Damage On Human Plasma, In Vitro.

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**Abstract:** This study investigates the role of buffalo colostrum and milk whey derived peptides in protection against oxidative damage induced by 2, 4-Dinitrophenol (2, 4-DNP) in human blood serum samples in vitro. A biomarker enzymes for oxidative stress like Alkaline phosphatase (ALP) and acid phosphatase (ACP), oxidative damage markers indicating extent of host antioxidant reserve indicators like reduced glutathione (GSH) were measured by spectrophotometric techniques in four different groups namely (1) Human blood plasma only (control) , (2) Human blood plasma + 2,4-DNP, (3) Human blood plasma + colostrum whey derived peptides + 2,4-DNP ,(4) Human blood plasma + colostrum whey derived peptides , (5) Human blood plasma + milk whey derived peptides + 2,4-DNP and (6) Human blood plasma + milk whey derived peptides. Following exposure to 2, 4-DNP, levels of antioxidants like GSH was significantly decreased in comparison to control, e.g., GSH [0.568 ± 0.015 vs 0.871 ± 0.022 μmol/0.1 mg proteins. In addition, the concentration of biomarker enzymes for 2,4-DNP induced membrane damage and oxidative stress like ALP and ACP were increased in serum by oxidant compared to control, e.g., ALP [5.497 ± 0.185 vs. 2.782 ± 0.000 μmol/mg protein], ACP [1.689 ± 0.047 vs. 0.629 ± 0.047 μmol/mg protein]. Pretreatment with buffalo whey derived peptides significantly protects 2,4-DNP induced RBC membrane lyses and release of ALP and ACP into serum environment. e.g., ALP [3.444 ± 0.094 vs. 5.497 ± 0.185 μmol/mg protein], ACP [0.629 ± 0.047 vs. 1.689 ± 0.047 μmol/mg protein]. Pretreatment with whey derived peptides give protection to oxidative damage and shifts the trend towards amelioration and replenishment of the antioxidant status.

**Keywords:** Buffalo Whey, peptide, Antioxidant, 2, 4-Dinitrophenol, Oxidative damage, GSH, ALP, and ACP.

## 1. INTRODUCTION

In recent years there is growing evidence that reactive oxygen species contribute to organ injury in many systems including the heart, liver and central nervous system [1]. Large amounts of ROS have been shown to participate in the pathogenesis of several human degenerative diseases, including inflammation, cardiovascular and neurodegenerative disorders, and cancer [2-4]. ROS also implicated in oxidative damage of macromolecules (lipids, proteins, DNA) [5]. Some of the main endogenous sources of free radicals are the mitochondrial respiratory chain, inflammation, peroxisomes and the cytochrome P450 [6]. Reactive oxygen species are constantly formed as by-product of normal metabolic reactions and their formation is accelerated by accidental exposure to occupational chemicals like pesticides. In respiring cells, the primary source of ROS is leakage of electrons from the mitochondrial respiratory chain [7-9]. In healthy individuals, the generation of reactive oxygen species (ROS) is well balanced by the counterbalancing act of antioxidant defenses. Hence an imbalance between ROS generation and antioxidant status in favor of the former has been described as oxidative stress [1].

Pesticides have been shown to have hepatotoxic and nephrotoxic effects. Organo-phosphorus insecticides may induce oxidative stress leading to generation of free radicals and hence an increased expression of oxygen free radical (OFR) scavenging enzymes in the target tissues [10, 11]. 2,4-Dinitrophenol is used in the manufacture of dyes, wood preservatives, and as a pesticide [12, 13]. During the 1930s, 2,4-dinitrophenol was used as a diet pill, but this use was stopped in 1938. Human Exposure to 2,4-dinitrophenol occurs from pesticide runoff to water and from releases to the air from manufacturing plants [14, 15]. The acute (short-term) effects of 2,4-dinitrophenol in humans through oral exposure are nausea, vomiting, sweating, dizziness, headaches, and loss of weight. Chronic (long-term) oral exposure to 2,4-dinitrophenol in humans has resulted in the formation of cataracts and skin lesions, weight loss, and has caused effects on the bone marrow, central nervous system (CNS), and cardiovascular system [16]. 2, 4-Dinitrophenol (DNP) is an uncoupler of oxidative phosphorylation in the mitochondria. It causes NADH loss, an increase in cytosolic Ca<sup>2+</sup> concentration and protein thiol loss [17]. DNP also caused a marked increase in apoptosis by DNA fragmentation (sub-G1 DNA content), increase the loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ), externalization of phosphatidylserine (PS). In addition, DNP-treated cells significantly increased the intracellular H<sub>2</sub>O<sub>2</sub>, induces ROS and reduce GSH content [18]. Living cells are naturally provided with an extensive array of protective enzymatic and non-enzymatic antioxidants that counteract the potentially injurious oxidizing agents. But even this multifunctional protective system cannot completely prevent the deleterious effects of reactive oxygen species (ROS), and consequently, molecules damaged by oxidation accumulate in cells. Restoration or activation of improperly working or repressed antioxidant machinery as well as suppression of abnormally

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amplified inflammatory signaling can provide important strategies for chemoprevention. Therefore, determination of anti-inflammatory and/ or antioxidant properties has been proposed as a good indicator for screening anti-cancer agents [19]. The cellular antioxidant pool comprises of integral antioxidants like glutathione and other thiols, and antioxidant free radical scavenging enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST). Long-term oxidative stress is also expressed as changes in extent of lipid peroxidation [20-22]. The cellular antioxidant action is reinforced by the presence of dietary antioxidants. Epidemiological surveys and laboratory investigations underline the utility of dietary agents that play an important role in inhibiting genotoxicity and carcinogenicity [23, 24]. Previous authors have shown that antioxidant food components have a protective role against oxidative stress-induced atherosclerosis, degenerative and age-related diseases, cancer and aging. Food derived peptides are promising natural antioxidants without marked adverse effects. In addition to their potential as safer alternatives to synthetic antioxidants used to avoid or retard oxidation reactions in foods, antioxidant peptides can also act reducing the risk of numerous oxidative stress-associated disorders [25-27]. Biologically active peptides derived from milk whey are initially found in inactive form within the sequence of the precursor molecules but it can be released in three ways; (i) enzymatic hydrolysis with digestive enzymes like pepsin, trypsin, chymotrypsin etc; (ii) fermentation of milk with proteolytic starter cultures; (iii) proteolysis by enzymes derived from proteolytic microorganisms [28]. Enzymatic hydrolysis disrupts the protein tertiary structure and reduces the molecular weight of the protein, enhancing the interaction of peptides with themselves and with the environment, and consequently altering their functional properties. Consequently, it is an effective means of liberating bioactive peptides from intact protein sequences [29, 30]. Antioxidant bioactive peptides were produced by *in vitro* enzymatic digestion of buffalo milk to study *in vitro* intestinal oxidative stress protection and anti-haemolytic capacity [31]. Enzymatic proteolysis (trypsin/chymotrypsin) of whey protein was produced effective antioxidant, antibacterial, immunomodulating, opioid and ace-inhibitory peptides [32-35]. There are four classes of biomarkers used to assess oxidative stress including measurement of radical production (i.e. OH $\cdot$ , O $_2\cdot$ ), AOs (i.e. glutathione (GSH), ascorbate), oxidation products (i.e. protein carbonyls) and the AO/pro-oxidant balance [36]. There is current interest in the development of therapeutic and chemo-preventive agents which have limited cytotoxicity. One potential approach would be to consider adding a non-cytotoxic adjuvant antioxidant to the pharmaceutical preparation. The implication of free radical mechanisms in the pathogenesis of human diseases and in the process of aging has continued to fuel suggestions that antioxidants, in particular milk protein derived bioactive peptides antioxidants, might have health benefits as prophylactic agents [37]. The present study has been initiated with the objectives of isolation of buffalo colostrum and milk whey protein from whole milk and to investigate antioxidant and potential protective effects of *in vitro* pepsin and pancreatin digested colostrum and milk whey protein hydrolysate on 2, 4-Dinitrophenol induced oxidative injury in

human serum; It also aimed to study the influence of digested milk and colostrum whey hydrolysate treatment on intracellular Glutathione (GSH) level, antioxidant activity.

## 2. MATERIAL AND METHODS

### 2.1. Materials and reagents

Pepsin P-7000, Pancreatin P1750, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS), acetic acid, sodium bicarbonate, potassium phosphate monobasic and dibasic, potassium phosphate dibasic, Sulfuric acid, HCl, Phenol, potassium chloride, BSA, NaOH,  $\beta$ -mercapthoethanol, o-phthaldialdehyde, SDS, GSH, disodium tetraborate, methanol, K $_2$ S $_2$ O $_8$ , Heparin, pNPP, EDTA, 2,4-DNP, formaldehyde, formic acid, tryptone were purchased from Sigma- Aldrich local supplier.

### 2.2. Collection of Milk and Buffalo samples

Unpasteurized Fresh buffalo milk and colostrum samples were obtained from local animal farm. All samples were transported to our laboratory on dry ice. Once in our laboratory, the samples were immediately frozen and stored at  $-18^{\circ}\text{C}$ . until ready for analysis.

### 2.3. Preparation of sweet whey from defatted whole milk samples

The frozen samples were thawed and the lipid fraction was removed by the method described by [38]. Briefly, Fresh buffalo milk and colostrum samples were centrifuged at 4,000 rpm for 30 min at  $4^{\circ}\text{C}$ . The upper cream or fat was removed and the liquid part, skimmed milk was transferred to new tubes. The skimmed milk and colostrums samples were dialyzed for 3 days against the distilled water with three changes per day using 10 kDa membranes. Equal volume of water was added to dialysate and decaseinated using acetic acid (pH 4.6, 2 N). The emulsion was centrifuged (8000 rpm, 30 min) at  $4^{\circ}\text{C}$  and the clear supernatant obtained was rich in acidic glycoprotein's referred as whey proteins, was then lyophilized and stored at  $-20^{\circ}\text{C}$  [39].

### 2.4. Determination of whey Protein and Sugar content.

Protein content of whey samples were determined by the Lowery's method [40], using bovine serum albumin as standard. In the case of Sugar, phenol-sulfuric acid method [41] were employed.

### 2.5. In vitro gastrointestinal digestion

*In vitro* digestion was carried out in duplicates according to the method described by [42]. Briefly, 3.5 % (w/v) whey solution in 0.1 M KCl-HCl (pH 2.0) buffer with pepsin (4% w/w) incubated for 4 h at  $37^{\circ}\text{C}$ . The reaction was stopped by keeping in boiling water for 10 min and neutralized to pH 7 using 2 N NaOH. The neutralized suspension (50 ml) was centrifuged (10,000 x g, 30 min) and the supernatant was used for estimation of peptides. The remaining neutralized suspension was digested further by 4% (w/w) pancreatin at  $37^{\circ}\text{C}$  for 4 h. The enzyme was inactivated by boiling for 10 min and centrifuged (10,000xg, 30 min), then the supernatant was used for different assay.

## 2.6 Estimation of peptide content

The peptide content of whey was measured by the method of Church et al. (1983) cited by [42]. 50 ml of reagent was prepared by mixing 25 ml of 100 mM borax, 2.5 ml of 20% (w/w) sodium dodecyl sulfate, 40 mg of o-phthalaldehyde solution (dissolved in 1 ml of methanol) and 100µl of β-mercapthoethanol and then adjusted to 50 ml with deionized water. 50µl of this whey hydrolysate was mixed with 2 ml Church's of reagent. The reaction mixture was incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured with spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The peptide content was quantified using casein tryptone as standard.

## 2.7 Collection of Blood and Serum and Isolation of Packed Erythrocytes.

Blood from healthy human was collected for experiments using Heparin (10 units/mL) as the anticoagulant. The collected blood was stored at 4°C and was used for experiments within four hours of collection. The serum samples were stored at -20°C and were used within 2 days of collection [43, 44]. The blood samples were centrifuged at 4°C for 10 min at 3000 rpm to remove plasma and buffy coat. The isolated erythrocytes were washed with normal saline (NaCl) thrice for packed erythrocytes.

## 2.8. In Vitro Experiments

### 2.8.1 ABTS radical-scavenging activity assay

The method described by [45] was used to measure the ABTS radical scavenging activity. The ABTS<sup>•+</sup> radical was generated through the addition of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> to ABTS. The solution obtained was further diluted with Phosphate Buffer Saline (PBS) to give an absorbance of 0.7 at 734nm. In brief, 50µl of each buffalo colostrum and milk derived peptides sample on the concentration of (0.25, 0.5, 0.75, 1, and 1.5 mg/mL) was mixed with 950µl diluted ABTS<sup>•+</sup> solution (absorbance of 0.70 ± 0.01 at 734 nm). The mixture was incubated in the dark for 6 min, and the absorbance at 734 nm was determined. The ABTS radical scavenging activity (%) was calculated as:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where A<sub>c</sub> is the absorbance of the control (ABTS<sup>•+</sup> solution without samples) and A<sub>s</sub> is the absorbance of the samples.

### 2.8.2. Suppression of 2, 4-Dinitrophenol-Induced Plasma Oxidation

#### 2.8.2.1. Determination of reduced Glutathione Concentration in human plasma (GSH)

The reduced glutathione (GSH) levels were measured fluorimetrically as described by [46] with minor modifications. Briefly, Human Plasma Samples (0.1 mg protein) were added to 1 ml formic acid (0.1 M) and centrifuged at 10,000g for 10 min. An aliquot of the supernatant was added to sodium phosphate buffer (0.1 M, pH 8.0, 5 mM EDTA) containing buffered formaldehyde (1:4 v/v, 0.1 M formaldehyde : 0.1 M Na<sub>2</sub>HPO<sub>4</sub>) and 0.1 ml of

O-phthaldehyde. The mixture was incubated for 45 min at 37°C and the fluorescence was measured at excitation and emission wavelengths of 345 and 425 nm, respectively. The concentration of plasma GSH was calculated using the standard curve and the values were expressed as µg GSH/mg protein.

### 2.8.2.2. Testing for antioxidant activity, Chemo-protection of colostrum and Milk whey derived peptides against chemically induced human plasma oxidation.

In this assay, the isolated human erythrocyte cells were exposed to the test compound, colostrum and milk whey hydrolysate, in physiological saline. The cells were allowed to absorb colostrum milk whey hydrolysate and any compounds not absorbed were removed by washing. These samples were used for experiments with or without treatment of 2, 4-DNP as plasma oxidative agent and the influence of colostrum and milk whey hydrolysate on plasma glutathione level of different experimental groups were quantified. In order to evaluate the effect of whey protein hydrolysates pretreatment on the GSH status and 2, 4-DNP, induced oxidative stress protection, an experimental groups were prepared as follow; Group-1: 0.1mg protein containing plasma (control), Group-2: 0.1mg protein containing plasma + 2, 4-DNP, Group-3: 0.1mg protein containing plasma + 2, 4-DNP + Buffalo colostrum whey hydrolysate. Group-4: 0.1mg protein containing plasma + Buffalo colostrum whey hydrolyzate, Group-5: 0.1mg protein containing plasma + 2, 4-DNP + Buffalo milk whey hydrolyzate and Group-6: 0.1mg protein containing plasma + Buffalo Milk whey hydrolyzate has been prepared.

### 2.8.2.3 Alkaline acid phosphatase plasma as oxidative biomarkers

The assays were done on the method described in [46, 47]. The alkaline phosphatase (ALP) activity was determined using p-nitrophenyl phosphate (pNPP; Sigma Chemical Co.) as substrate. The latter was dissolved in 0.05 M Tris buffer, pH 9.5, and enzyme samples were incubated with substrate at 37°C for 30 min. Specific activity was determined as the number of micromoles of pNPP liberated per minute per milligram of soluble protein. Quantitation of protein was determined by the procedure of Lowry[40]. ACP was determined similarly as for ALP, using the same reaction mixture, except that pNPP was dissolved in 0.05 M sodium acetate buffer, pH 5.0, which served as the reaction buffer.

## 3. RESULTS AND DISCUSSION:

Pale yellow to golden color colostrum of buffalo and their respective mature milk was defatted followed by isoelectric precipitation of casein to obtain whey proteins.



### 3.1: Quantification of buffalo colostrum & milk whey proteins

**TABLE 1: TOTAL PROTEIN & SUGAR (G/L) IN BUFFALO COLOSTRUM & MILK WHEY**

	Buffalo	
	Colostrum	Milk
Dialyzed Whey Protein content (g/l)	192.4	6.5
Yields (%)	20	1.4
Dialyzed Whey Sugar content (g/l)	1.08	0.06

The amount of whey protein and sugar in each sample was quantified as shown in table 3.1. The amount of protein in buffalo colostrum whey was found to be 194.2 g/l which is almost similar with 1st day camel colostrum whey protein content ( from 198 to 118 g/l in 24 h) found in [48] research paper. On the other hand, the respective buffalo milk whey protein concentration was found to be 6.5g in a liter of milk. In comparison to buffalo colostrum colostrum which is having (194.5g/l) whey protein , buffalo milk whey showed less protein content (6.5g/l). The content of milk whey protein found in the study 6.5g/l are similar with that of buffalo whey protein content investigated by S Rafiq and his research collogue [49]. Further, the whey protein content of buffalo milk was also found less to that of a documented literature data of 9.7g/l of goat whey protein [50]. Similarly , whey proteins of buffalo (6.5g/l) found in these study is higher than cow's milk whey protein (4.5g/l) reported previously reported in Masud paper [51]. This variation in milk whey could be due to the environmental conditions, feed, stage of lactation, season of lactation, parity, genetic makeup, breed, etc. [52]. The content of sugar was found to be higher in a colostrum sample in analogy to respective milk samples. This can be correlated with the earlier research report of [53], who have identified lower carbohydrate content in milk compared to colostrum. In this context more studies has to be carried out on to characterize glycol-conjugates present in colostrum whey.

### 3.3. Proteolytic digestion of whey samples

Protein containing samples from buffalo colostrum and milk wheys were treated in vitro with gastro-intestinal proteinase enzymes namely, pepsin and pancreatin. The peptide content after proteolytic in vitro digestion were measured by the method of church [54]. From the table-2, it can be observed that high yield of peptides was evident in double digested samples. Peptide concentration for buffalo colostrum whey varied from 1537 to 7459  $\mu\text{g}/10\text{mg}$  of the total protein during double (pepsin / pancreatin) digestion. On the other hand, the peptide concentration for single digested buffalo colostrum and milk whey pepsin digested sample found to be between 1988.4 & 680  $\mu\text{g}/10\text{mg}$ , respectively. Among the buffalo whey's derived peptides; buffalo colostrum whey produced larger concentration of peptides, 7459  $\mu\text{g}/10\text{mg}$ , compared to milk whey proteins. Moreover, milk hydrolysate gave low yield of peptides, 680  $\mu\text{g}/10\text{mg}$  and 1537  $\mu\text{g}/10\text{mg}$  for single and double digested buffalo milk whey hydrolysate , respectively

**TABLE 2: CONCENTRATION OF PEPTIDES AFTER PROTEASE TREATMENT IN COLOSTRUM AND MILK WHEY PROTEIN SAMPLES**

S. No	Sample s	Abs.	Conc. ( $\mu\text{g}$ ) form 1mg whey	Yield (in $\mu\text{g}/10\text{mg}$ )
1	P-BC	0.696	198.84/20 $\mu\text{l}$	1988.4
2	P-BM	0.241	68.86/50 $\mu\text{l}$	680
3	P/P-BC	2.61	745.87/20 $\mu\text{l}$	7458.7
4	P/P-BM	0.538	153.71/50 $\mu\text{l}$	1537.1

**Note:** P-BC & P-BM: Pepsin digested buffalo colostrum whey & Buffalo milk whey, P/P-BC & P/P-BM: Pepsin/Pancreatic Double digested buffalo colostrum & buffalo milk whey.

Pepsin, being one of the principal protein degrading enzymes, is most efficient in cleaving peptide bonds at N-terminal between hydrophobic & preferably aromatic acids such as phenylalanine, tryptophan and tyrosine. But lower yield of peptides after pepsin digestion was attributed to resistant whey proteins like  $\alpha$ -lactalbumin ( $\alpha$ -La) and  $\beta$ -lactoglobulin ( $\beta$ -Lg), which takes high retention time for digestion, and significantly resists pepsin attack. The resistance could also be due to the presence of 4 and 2 intermolecular disulfide bridges of  $\alpha$ -La and  $\beta$ -Lg, respectively. However, pancreatin (a mixture of several proteases) hydrolyzes different whey proteins into oligopeptides. Therefore, pepsin-pancreatin treatments are expected to give higher quantity of peptides when compared to their respective single digested whey protein samples. Thus, the P/P of colostrum whey proteins yielded higher quantity of peptides, compared to peptides generated by pepsin (P) alone [55].

### 3.4. In Vitro Experiments

#### 3.4. Antioxidant activities of buffalo colostrum and milk whey derived peptides

##### 3.4.1. ABTS antioxidant activities of whey derived peptides

##### A. ABTS antioxidant activities of Double digested( Pepsin & Pancreatic) colostrum and milk whey

Antioxidant activities of (P) and (P/P) of buffalo milk and colostrum whey derived peptides were carried out for ABTS assay using ABTS as free radical substrate.

**TABLE 3: MEAN ANTIOXIDANT ACTIVITIES OF PEPTIDES FROM BUFFALO COLOSTRUM AND MILK WHEY DERIVED PEPTIDE SAMPLES.**

Pepsin + Paecreatin Digested Buffalo Colostrum and Milk whey	% of antioxidant activities of five concentration				
	0.25 mg/ml	0.5mg/ml	0.75mg/ml	1mg/ml	1.5mg/ml
Buffalo Colostrum	31	38	47	50	53
Milk	23	24	31	30	34

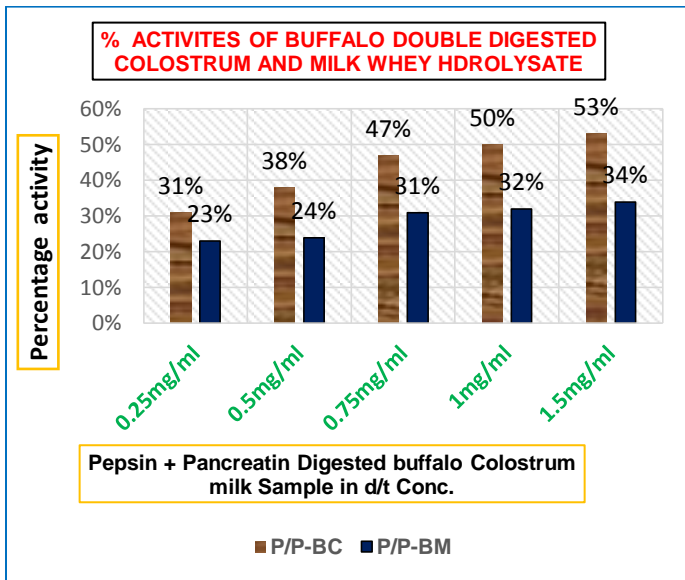


Figure 1: Percent antioxidant activities of double digested (P/P) samples.

Varied concentrations of peptides (0.25 - 1.5mg /ml) from buffalo colostrum and milk samples were analyzed. As shown in Fig. 4, colostrum samples exhibited higher order of radical scavenging activity. Among the five buffalo colostrum derived peptide used concentrations, [1.5mg/ml] peptide concentration showed highest ABTS radical scavenging activity (53%) and in contrary, less (31%) ABTS radical scavenging activity showed at [0.25mg/ml] concentration. Milk whey peptides on the same concentration with colostrum whey peptide used and the highest percentage of antioxidant [34%] were showed on 1.5mg/ml milk whey derived peptides. On comparison with double digested colostrum whey (31%-53%), Milk whey drived peptides were showed relatively lower percentage antioxidant activities (23% -34%). From the above study, percentage antioxidant activities of buffalo colostrum whey derived peptide, the degree of antioxidant activities by the whey derived peptides were known to be dose dependent.

**B. ABTS antioxidant activities of Single (Pepsin) digested colostrum and milk whey**

From the study, it is be able to know that single digested samples , pepsin digested samples at the concentration of [0.25mg/ml] and [0.75mg/ml] showed lower antioxidant activity of 29% , 44% for buffalo colostrum and 27% ,31% for milk whey peptides , respectively. The result showed percentage of antioxidant activities of whey protein increases with increased degree proteolysis by enzyme.

TABLE 4: MEAN PERCENTAGE OF ANTIOXIDANT ACTIVITIES FOR PEPSIN DIGESTED WHEY SAMPLES IN THE CONC. OF 0.25MG/ML AND 0.75MG/ML.

Pepsin digested Samples	% of antioxidant activities in 0.25 and 0.75mg/ml concentration	
	0.25mg/ml	0.75mg/ml
Buffalo colostrum	29	44
Milk	27	31

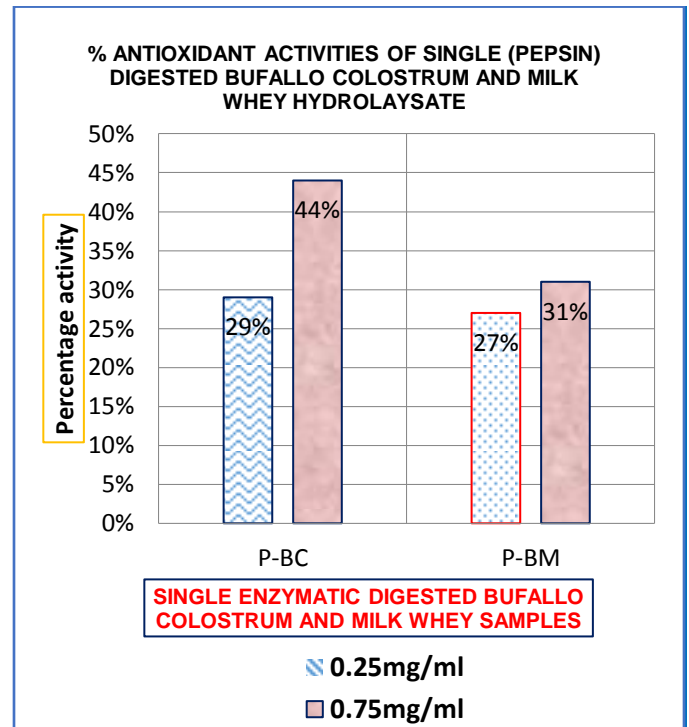


Figure 2: Percent activities of single digested (P) samples

The higher antioxidant activity of double digested whey protein hydrolyzates compared to single digested whey may be resulted from the differences in amounts and/or in accessibilities of antioxidant amino acid residues present in their primary structures. Although, antioxidant mechanism of peptides is not fully understood, peptides containing histidine residues have been demonstrated to act as metal-ion chelators, active-oxygen quencher and hydroxyl radical scavenger [56]. The ability of protein hydrolyzates to inhibit deleterious changes caused by lipid oxidation appears to be related to the nature and composition of the different peptide fractions produced depending on the protease specificity. [29, 56].

**3.5. Suppression of 2, 4-Dinitrophenol-Induced Plasma Oxidation**

**3.5.1. Determination of reduced Glutathione Concentration in human plasma (GSH)**

The reduced glutathione (GSH) levels were measured fluorimetrically as described by [46] with minor modifications. The concentration of plasma GSH was calculated using the standard curve and the values were expressed as µg GSH/mg protein.

**3.5.2. Testing for antioxidant activity, Chemo-protection of colostrum and Milk whey derived peptides against chemically induced human plasma oxidation.**

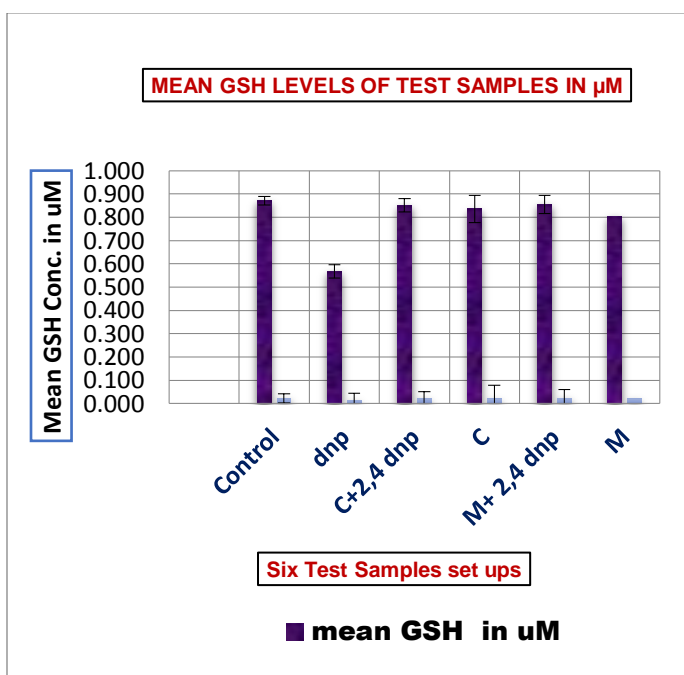
Glutathione (GSH) is a tripeptide composed of cysteine, glutamic acid and glycine. GSH has two characteristic structural features: γ-glutamyl linkage and a sulphhydryl (-SH) group. GSH is known to have multifaceted physiological functions, plays an important role in the detoxification of xenobiotic compounds and in the anti-

oxidation of reactive oxygen species (ROS) and free radicals [57].

**TABLE 5: MEAN CONCENTRATION OF GLUTATHIONE (GSH)**

Experimental Group	Treatment	GSH (Glutathione), Mean Conc. ( $\mu\text{M}/0.1\text{mg}$ serum protein) $\pm$ SD
Group-1	Control (0.1mg protein Serum)	0.871 $\pm$ 0.022
Group-2	DNP + 0.1mg protein Serum	0.568 $\pm$ 0.015
Group-3	BCWH+2,4 DNP + 0.1mg protein Serum	0.852 $\pm$ 0.022
Group-4	BCWH + 0.1mg protein Serum	0.836 $\pm$ 0.020
Group-5	BMWH + 2,4 DNP +0.1mg protein Serum	0.855 $\pm$ 0.023
Group-6	BMWH + 0.1mg protein Serum	0.805 $\pm$ 0.021

**Note: BCWH-Buffalo Colostrum whey Hydrolysate , BMWH:Buffalo Milk whey Hydrolysate, DNP: 2, 4-dinitrophenol**



**Figure 3: Concentration of GSH ( $\mu\text{M}$ ) for the experimental set ups.**

In order to evaluate the effect of whey protein hydrolysates on the GSH status after exposure to 2, 4-DNP, a known oxidative stress creating chemical, an experiment was set up by mixing, Group-1: 0.1mg protein containing serum (control), Group-2: 0.1mg protein containing serum + 2, 4-DNP, Group-3: 0.1mg protein containing serum + 2, 4-DNP + Buffalo colostrum whey hydrolysate. Group-4: 0.1mg protein containing serum + Buffalo colostrum whey hydrolysate, Group-5: 0.1mg protein containing serum + 2,4-DNP + Buffalo milk whey hydrolysate and Group-6: 0.1mg protein containing serum + Buffalo Milk whey hydrolysate has been prepared. Result analysis from Table-5 and figure-3 above revealed, the Glutathione (GSH) content of Group-1 (Control) was found  $0.871 \pm 0.022/0.1$  mg serum protein for experimental sample under study.

The 2,4-Dinitrophenol added samples was enzymatic alteration induced and resulted in a depleted serum GSH to significantly greater extent;  $0.568 \pm 0.015 \mu\text{M} / 0.1\text{mg}$  protein for the sample having 0.1mg serum protein + toxic 2,4-DNP without treatment of colostrum and milk whey hydrolysate, after 45 minute exposure. However, treatment of the serum sample containing 2,4-DNP with colostrum/milk whey hydrolysate or colostrum / milk whey hydrolysate only did not have significant change in GSH content. The 45 Minutes exposure of 2, 4-DNP treated serum sample with colostrum / milk whey hydrolysate, resulted in inhibition or chemo protection from toxic effect raised by 2,4-DNP. Moreover, treatment of the test group with milk/colostrum whey hydrolysate promoted the synthesis of GSH and ultimate increment of GSH as showed in the (Group-5) in vitro study. This study clearly revealed that in vitro treatment of whey protein hydrolysate, increased the concentration of Glutathione by supplying component of glutathione, cysteine amino acids. The study clearly revealed that the in vitro treatment of whey protein hydrolysates increased the GSH level to  $0.919 \mu\text{M}/0.1\text{mg}$  protein and masked the effect 2, 4-DNP, free radical forming chemical. Milk and colostrum hydrolysates known to have high amount of cysteine, the amino acid component of GSH, and also it is called rate limiting factor in the glutathione synthesis. The addition of whey hydrolysate boost the production of glutathione which in turn helps in protection oxidative deterioration of the cell by Scavenging free radicals, production and repairing of DNA and in folding of newly synthesized proteins [58, 59].

### 3.5.1. The Effect of Whey Hydrolysate on GSH

In the DNP treated samples, Group-2, Group-3 and Group-5, there was drastic decline of glutathione by [0.303], [0.019] and [0.016 $\mu\text{M}$ ] in serum, respectively compared to control [0.871 $\mu\text{M}$ ]. The highest, [0.303 $\mu\text{M}$ ] concentration of glutathione was decrease due to oxidative stress created by dinitrophenol (DNP) treatment in the group-2 in vitro experimental set up. This decreased level of glutathione concentration again increased to [0.284  $\mu\text{M}$ ], [0.287  $\mu\text{M}$ ] when treated with colostrum and milk whey hydrolysates, respectively.

### 3.5.2 Buffalo colostrum and milk whey derived protection against GSH oxidation

In the experiments using GSH as the target of oxidation, we found that 2, 4-DNP caused a strong oxidation of the GSH thiol group thus leading to a significant reduction in GSH concentration in the reaction mixture ( $0.568 \mu\text{M} / 0.1\text{mg}$  serum protein (approximately a reduction of 35% with respect to control ( $0.871 \mu\text{M}$  GSH /  $0.1\text{mg}$  serum protein)). When oxidation occurred in the presence of buffalo colostrums and whey derived peptides at 1mg/ml, we observed that whey derived peptides chemo-protected and inhibited the oxidation of GSH induced by 2, 4-DNP (Figure-3). The best protection against thiol group oxidation was observed when buffalo milk and colostrum whey derived peptide used as pretreatment; moreover, it showed intrinsic increased the total GSH level by 3% when compared to control ( $0.871 \mu\text{M}$  GSH/ $0.1\text{mg}$  protein). The pretreatment of buffalo colostrum whey derived peptides on the chemically induced oxidative stress plasma sample showed ( $0.852 \mu\text{M}$  GSH/ $0.1\text{mg}$  serum protein), 33.3% protection against



oxidation of GSH thio group by 2, 4-DNP treated serum sample (0.568  $\mu\text{M}$  GSH/0.1mg serum protein). The protection capacity of whey protein hydrolysate on H<sub>2</sub>O<sub>2</sub>-induced PC12 cells oxidative stress also showed in the previous study [60]. In these study, it was observed that whey derived peptides inhibited the oxidation of GSH induced by the 2, 4-DNP, oxidants, thus maintaining GSH thiol groups in their reduced state. GSH is the most abundant non-enzymatic antioxidant present in mammalian cells and plays a very important role in maintaining cellular redox homeostasis [61]. During oxidative stress, the cellular pool of GSH is depleted, consequently, exogenously introduced radical scavengers, such as milk or colostrum whey peptides, may protect GSH levels in cells by preventing them from being consumed in reactions with free radicals [6]. We also evaluated the antioxidant capacity of whey derived peptides using ABTS assay. Our experiments indicate a high protection of buffalo milk whey derived towards GSH oxidation, especially when induced by the radical generating system 2,4-DNP. These radical species are mainly produced in mitochondria [62] and it has been demonstrated that the oxidative damage to mtDNA is implicated in physiological senescence and age-related disorders such as sporadic neurodegenerative disorders, type II diabetes, cancer and cardiac diseases [63, 64]. Our results suggest that colostrums or milk whey derived supplementation might play a role in mitochondria viability and the long term consumption of milk may be helpful in diseases related to mitochondrial dysfunction. Being particularly susceptible to oxidative damage, erythrocytes were also chosen as a model system for investigating whey peptides antioxidant properties. RBC serums have a high cellular concentration of Hb and are directly exposed to molecular oxygen. At the same time, they have high polyunsaturated fatty acid content in their membranes making them highly susceptible to peroxy radical mediated oxidation and a decrease in intracellular GSH levels [65]. In our experiments, we found that whey derived peptides effectively protected serum GSH depletion induced by 2, 4-Dinitrophenol, thus furnishing protection against oxidative and preserving the endogenous antioxidant defense system.

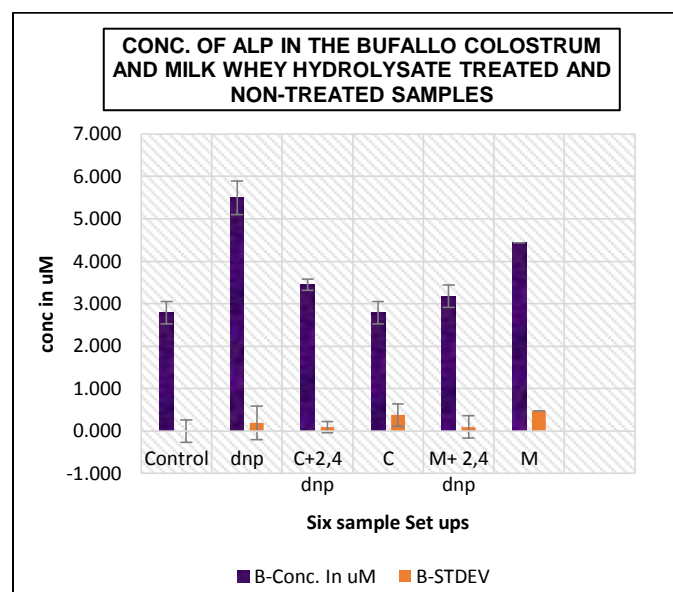
### 3.6. Plasma Alkaline and acid phosphatase, oxidative biomarkers

**TABLE 6: THE CONCENTRATION OF ALKALINE PHOSPHATASE**

Experimental Groups	Treatment	Mean ALP Conc. in $\mu\text{M}/\text{mg}$ of serum and St. Dev.
Group-1	Control (0.1mg protein Serum)	2.782 $\pm$ 0.000
Group-2	DNP + 0.1mg protein Serum	5.497 $\pm$ 0.185
Group-3	BCWH+2,4 DNP + 0.1mg protein Serum	3.444 $\pm$ 0.094
Group-4	BCWH + 0.1mg protein Serum	2.782 $\pm$ 0.375
Group-5	BMWH + 2,4 DNP +0.1mg protein Serum	3.179 $\pm$ 0.094
Group-6	BMWH + 0.1mg protein Serum	4.438 $\pm$ 0.468

**Note:** BCWH-Buffalo Colostrum whey Hydrolysate, BMWH: Buffalo Milk whey Hydrolysate, DNP: 2, 4-dinitrophenol

Exposure of RBC serum to oxidant, Dinitrophenol resulted in the loss of intracellular GSH that precedes oxidation of membrane thiols and the formation of oxidative radicals. Moreover, when RBC serum exposed to 2,4-DNP, it causes an immediate change in the RBC membrane structure, damages to serum components consequently RBC cell releases its membrane component, Alkaline phosphatases (ALP) and Acid phosphatases (ACP) which are biomarkers for oxidative stress. The research studied the effect of 2, 4-DNP on the concentration biomarker for oxidative stress enzymes concentration; ALP and ACP in the serum in vitro. In our experiments, we found that buffalo colostrums whey derived peptides promptly reduced hemolytic effect of 2, 4-DNP, release of biomarker enzyme to serum and GSH depletion induced by DNP.



**Figure 4: Concentration of ALP in  $\mu\text{M}$**

The experimental set up was done as described in GSH assay. The samples were mixed with substrate and the level of hydrolysis of p-NPP to NP was measured as the activities of alkaline phosphatase with the released inorganic phosphate. The present study showed the effect of DNP and different milk and colostrum whey hydrolysate on the activities of alkaline phosphatase. The presence of different radical forming chemicals affects the physiology of living cells. The levels of effect of DNP, whey hydrolysate +DNP, Milk/colostrum whey hydrolysate of different animals have been shown in Figure 4. The result clearly demonstrated that the 2, 4-DNP increased the level of serum Alkaline phosphatase. Alkaline phosphatase (Figure-4), oxidative biomarkers was observed to be elevated by 1.97 fold in the DNP treated group compared to control group. Surprisingly, colostrum and milk whey hydrolysate treated serum samples, reduced the level of alkaline phosphatase offering protection against injurious chemical effect.

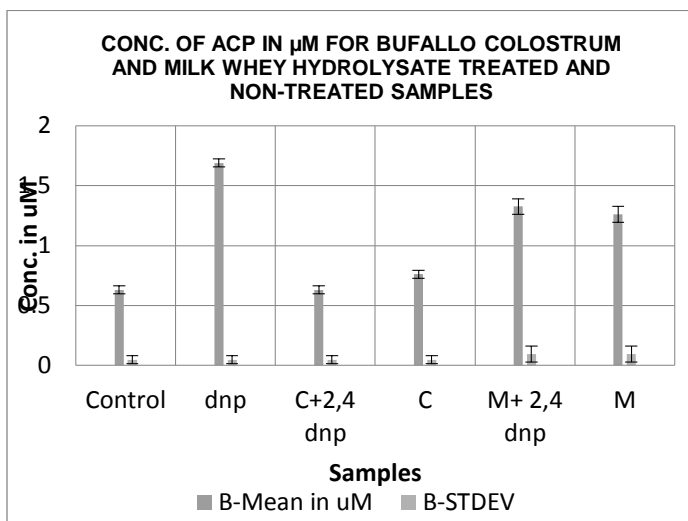
### 3.7. Acid Phosphatase (ACP) Assay

**TABLE 7: MEAN CONCENTRATION OF ACID PHOSPHATASE**

Experiment al Groups	Sample Set ups	Mean ACP Conc.( $\mu\text{M}$ ) of each samples $\pm$ St. Dev
Group-1	Control (0.1mg protein Serum)	0.629 $\pm$ 0.047
Group-2	DNP + 0.1mg protein Serum	1.689 $\pm$ 0.047
Group-3	BCWH+2,4 DNP + 0.1mg protein Serum	0.629 $\pm$ 0.047
Group-4	BCWH + 0.1mg protein Serum	0.762 $\pm$ 0.047
Group-5	BMWH + 2,4 DNP +0.1mg protein Serum	1.325 $\pm$ 0.094
Group-6	BMWH + 0.1mg protein Serum	1.258 $\pm$ 0.094

**Note:** BCWH-Buffalo Colostrum whey Hydrolysate ,  
BMWH:Buffalo Milk whey Hydrolysate, DNP: 2, 4-dinitrophenol

The acid phosphatase test measures the level of acid phosphatase in the blood serum. Acid phosphatase is essentially a type of enzyme, which hydrolyses phosphate ester bonds under an optimum pH of 4.8 and temperature of 37 °C. In the present study showed, the in-vitro DNP induced oxidative stress increased plasma level acid phosphatase enzymatic activities by 2.84 $\mu\text{M}$ -fold compared to the control i.e. 0.629 $\pm$ 0.047  $\mu\text{M}$ .



**Figure 5: Concentration of ACP in  $\mu\text{M}$**

This represents that under normal physiological condition, acid phosphatase present in the serum shows lower activity, when compared with presence of toxic molecules like, 2,4 DNP, which induces the oxidative stress in the serum & affects the rate of enzyme i.e., increasing activity. This assay has also shown that the addition of colostrum whey hydrolysates to the tube containing 2,4-DNP plus blood plasma sample, resulted in decreased activity of enzyme, giving the value i.e. 0.629  $\pm$  0.047, which is lesser than the respective milk hydrolysate containing tube, with the values showing 1.258  $\pm$  0.094 $\mu\text{M}$ . This variation in the enzymatic activity showed that the colostrum whey -derived peptides have better capability of restoring the pathophysiological condition of serum than its respective milk whey-derived peptides. The present study clearly

showed that the buffalo, colostrum hydrolyzate , as well milk hydrosylates are playing important role in restoring the activity of alkaline and acid phosphatases, which might be because of binding of these short peptides to the specific docking sites present on the enzyme, which might have changed its specific configuration & its active site & thus showing the decreased activity [46].

### 4. CONCLUSIONS

In the present study we have clearly evidenced that, whey protein hydrolysates were the potential protector against induced oxidative stress. Our data confirmed that whey protein hydrolysates as valuable antioxidant constituents may be produced by proteolytic degradation during in vitro gastro intestinal digestion. They did this via their effect on increasing GSH concentration in the serum. It is considered that oxygen radical generation or oxidative stress in the body is frequently a critical step in disease condition. Hence the effect of GSH on free radicals and supplementation of whey protein hydrolysates, could be important in inhibiting carcinogenesis induced by a number of different mechanisms. The study results indicated that supplementation of cysteine-rich whey protein isolate leads to increased plasma GSH protection from oxidative stress. Whey bioactive peptides can be effectively used in health-promoting foods as a bio-functional ingredient.

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