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Bread (composite flour) formulation and study of its nutritive, phytochemical and functional properties

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Abstract
Grains are a rich source of nutrition and phytochemical compounds which make them a good source of antioxidants. These phytochemicals have potential impact on human health. The present study deals with formulating medium strength, refined and whole wheat flour using sorghum, buckwheat, chickpea, sprouted wheat and sprouted barley and screening their enriched nutritional and phytochemical profile. Four different formulations were prepared with composite multigrain mix flour. Out of which two composite formulations were opted for further analysis. Additional flours were taken to improve bread's crumb structure (corn and defatted soy flour). Nutritional analysis indicates better nutritive profile having more protein, fiber with low fat, moderate carbohydrate value and high mineral contents. Besides nutritive value, phytochemical analysis also revealed composites flours and products to be a good source of phenolics (11.8-17.87 µg GAE/ mg sample), flavonoids (21.6-106.4 µg QE/ mg sample), and antioxidant.
Comparative study for evaluating the effect of high baking temperature (200 ℃), extraction of the final accepted bread was also performed and phytochemical analysis was done on them. The study revealed that not all phenolics and flavonoids are heat sensitive. Chromatography techniques like HPLC and GC-MS was also performed on raw composite flour and on bread extract. HPLC analysis revealed the presence of flavonoid compounds like rutin, quercetin, epicatechin and chlorogenic acid. GC-MS analysis shows the presence of hexadecanoic acid, vitamin E, stigmasterol and many other secondary metabolite compounds which contributes to high antioxidant potential and other significant therapeutic uses, in both the composite extracts. The effects of composite flours on physical dough properties and bread structure were studied. The bread loaves have been produced using the straight dough method. Physiochemical and physical characteristics of the bread like loaf height, loaf volume, loaf weight and baking loss were also estimated.

Keywords: Composite flour, Bread, Phytochemical, GC-MS, HPLC, Physicochemical.

Introduction
Since consumers, nowadays, are more concerned about their health, they focus on consuming products which boost up their immune systems. Food with high protein and fiber content are now mostly preferred by consumers to maintain their health and keep them away from many types of diseases like cardiovascular disease, diabetes, weight gain, etc. So there is a new trend in the market to develop a product that combines the health benefits with good sensory properties.
Bread is one of the oldest and largest consumed foodstuffs and is consumed across the globe by all age groups, the present study, therefore, aims to formulate a functional or healthy bread. Bread may be described as a fermented confectionery product which is produced mainly from wheat flour, yeast, water, sugar, salt and other ingredients needed accordingly, by a series of process involving mixing, kneading, proofing, shaping, baking [1]. Bakery products like bread and other bakery goods like biscuit, cake, muffins, etc. made from wheat flour is very popular but has low protein content. Whole grains were preferred in the present experiment as fiber present in outer bran part of the grain has many health benefits. According to epidemiological studies the consumption of whole grains products attributes to reduce the risk of oxidative-stress related chronic diseases and age related disorders, such as cardiovascular diseases, carcinogenesis, type II diabetes and obesity [2].
In the present study, composite flour bread was prepared using refined wheat flour, whole wheat flour, whole grain buckwheat flour, whole grain desi chickpea flour, whole grain sorghum flour, sprouted wheat and sprouted barley flour with an aim to formulate an enriched flour which is high in protein, fiber content. Additional flours were taken to improve bread's crumb structure (corn and defatted soy flour). These flours not only increase the bread rheology, but also increase the nutritional value of the product.

Flax seeds and muskmelon seeds were sprinkled over the final prepared dough to give it an appealing texture. Besides nutrition, consumers are more attracted by flavor, texture and aroma of the product therefore these attributes are kept in mind during the formulation of composite flours and maintained by using 60% whole wheat or refined wheat flour and 35% multigrain mix flours for bread making. Bread with multigrains is the healthiest option since they add extra nutrition, protein, fiber, minerals and vitamins. These grains not only increase the nutritive valve, but also increase the phytochemical characteristics of the bread.

Wheat (*Triticum aestivum*) is the principal crop used for bread making. Many epidemiological studies have been carried out on consuming whole wheat and indicated that whole wheat foods could reduce the risk of cancers [3, 4], diabetes [5, 6] and coronary heart disease [7, 8]. Barley (*Hordeum vulgare*) is considered as a functional and healthy grain. It is a rich source of soluble fiber, beta-glucan. According to Food and Drug Administration soluble fiber reduces the risk of CHD based on their effect on total and LDL blood cholesterol. Also beta-glucan helps in maintaining the blood sugar level and prevention of diabetes. Buckwheat (*Fagopyrum esculentum)* is a Pseudocereals grain, non-grass family. It is a fruit seed and does not contain gluten, therefore, make it suitable for people sensitive to wheat gluten. The biological function of buckwheat was reviewed such as antimutagenicity, anticarcinogenicity and anti aging [9]. Buckwheat phenolic compounds such as rutin, phenolic acid, 3-flavanols and their derivatives have stronger antioxidative activity than oats and barley [10]. Another grain used in the study is a legume, chickpea (*Cicer arietinum*) which provide many health benefits components as rich in fiber, protein for vegetarians, manganese for energy production, boost iron, stabilizes blood sugar and have low glycemic index (GI) and regular intake of chickpeas can lower LDL (bad) and total cholesterol. In this study Desi chickpea is used for bread preparation. One of the major components of chickpea is saponin which has an ability to form an insoluble complex with cholesterol in our body and act as a cholesterol lowering agent. Sorghum *(Sorghum bicolor)* is native to Africa, but can now be found all around the world as a staple food product. It inhibits cancer tumor growth, protect against diabetes and insulin resistance, it helps to manage cholesterol and help in treating human melanoma.

Sprouting of two grains, i.e. wheat and barley were performed. Sprouting increases the protein digestibility and also decreases the anti-nutritive materials (trypsin inhibitor, phytic acid, pentosan, tannin). Phytic acid combines with key minerals, especially calcium, magnesium, copper, iron, and zinc and prevents their absorption in the intestinal tract. Fermentation, baking and sprouting decreases its content. Additional flours used were defatted soy flour and corn flour just to increase the final dough rheology.

The objectives of the research were to prepare the healthy bread, enriched with protein and fiber, from opted composite flours formulations, to evaluate nutritional, phytochemical activity, functional and physiochemical properties of the flours and breads. Also to identify the anti-diabetic and other health beneficial compounds in the composite flour by chromatography method and evaluating the effect of heat on these identified compounds.

2. Materials and methods

2.1 Collection of Raw Materials

All the grain (wheat, sorghum, buckwheat, desi chickpea and barley) was purchased from the Najafgharh local market, located at the outskirts of the southwestern part of Delhi, India.

2.2 Reagents

Acetic acid, aluminium chloride, ascorbic acid, catechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, Folin Ciocalteu's phenol reagent, gallic acid, sodium acetate, sodium carbonate, sodium nitrite, 2,4,6-Tripyridyl-s-triazine (TPTZ), hydrochloric acid, n-butanol, sodium hydroxide, tannic acid, potassium ferrocyanide, methanol, ethanol, diethyl ether, BHT, DMSO, potassium di-hydrogen orthophosphate, milli Q water, acetonitrile, hydrochloric acid, benzene, chloroform, ammonium hydroxide, potassium hydroxide, nutrient agar, nutrient broth, conc. H_2SO_4.

2.3 Sprouting of grains

There are several ways to sprout seeds, the most common is the "soak, rinse and drain" method. Desired grains were sprouted by this method for 4 – 5 days. After drying sprouted grains were grounded and stored in an airtight jar.

2.4 Preparation of Composite flours for bread preparation

The whole grains, seeds were cleaned from dirt by sorting out contaminants such as sand, sticks and leaves and washed and oven dried. Later all grains were milled and sieved properly. The flour was stored in airtight containers until further use. All the flours were sieved through 180 micron sieve for finer particles.

The experimental variations of composite flours were formulated using refined wheat flour, desi chickpea flour, buckwheat flour, sorghum flour, sprouted wheat flour and sprouted barley flour. Additional flours were also used like corn and defatted soy flour for better dough rheology. Four different proportions of composite flours were used are- 1) 50% refined wheat, 10% chickpea, 10% buckwheat, 10% sorghum,10% sprouted barley and 10% sprouted wheat (MI), 2) 50% whole wheat, 10% chickpea, 10% buckwheat 10% sorghum, 10% sprouted barley and 10% sprouted wheat (MII), 3) 65% refined wheat, 5% chickpea, 5% buckwheat 5% sorghum, 5% sprouted barley, 5% sprouted wheat, 5% corn flour and 5% defatted soy flour (RWI), 4) 65% whole wheat, 5% chickpea, 5% buckwheat, 5% sorghum, 5% sprouted wheat, 5% sprouted barley, 5% corn flour and 5% defatted soy flour (RWII).

2.5 Bread preparation by Straight-dough method

The straight dough method is the easiest of the dough-making methods where all the ingredients are mixed at the same time in the mixer. Firstly the water, compressed yeast and sugar were mixed properly in a separate bowl. And was left for 10-15 min till it forms slump and bubbles, this indicates that yeast is activated. All the flours were mixed with water (60% w/w) along with sugar-yeast solution and other required ingredients (salt, gluten, hydrocolloid, improver, preservative, dry milk powder) in a planetary mixer to form homogeneous dough. During mixing the hydration was checked. 5-7 inches the dough was stretched to check the proper gluten formation.

After proper kneading for 10-12 mins, the dough was manually punched and left for 10-15 min in bench, this is called bench rest where first fermentation takes place. In between the fermentation the dough was knock backed to expel the excess gas and to redistribute the food for the yeast. Usually the knock-back is done when 2/3 of the fermentation time is over. Then the dough was transferred to previously oiled molder so that dough does not stick to molder. This was placed in proofer at temperature 35-36℃ for 50-55 min (since multi-grains need more time for proofing) and 85% relative humidity. Then the proofed dough was placed in a preheated (210℃/200℃) deck oven for baking for 45-50 min. After baking the IPT (internal product temperature) of formulated breads were measured.

Table 1: - Mixture contents of different formulated breads (CP-chickpea, S-sorghum, BW- buckwheat, SWT-Sprouted wheat, SBA- sprouted barley, CF- corn flour, and DS-Defatted soy)

Ingredients	Control	Formulation 1(gm)(MI)	Formulation 2(gm)(MII)	Formulation 3(gm)(RWI)	Formulation4(gm) (RWII)
Compressed yeast (g)	3	4	4	4	4
Salt (g)	1.5	2	2	2	2
Sugar (g)	4	6	6	6	6
Water (ml)	60	60	60	60	60
Dry milk (g)	-	7	7	7	7
Refined Wheat Flour (g)	100	50	-	65	-
Whole Wheat flour (g)	-	-	50	-	65
Added flour (g)	-	50g(10gCP: 10gS:10gBW:10gSWT: 10gSBA)	50g(10gCP:10gS:10gBW:10gSWT:10gSBA)	35g(5gCP:5gS:5gBW:5gSWT:5gSBA:5g CF:5gDS)	35g(5gCP:5gS:5gBW:5gSWT:5gSBA: 5g CF:5gDS)
SSL Emulsifier (g)	-	-	-	0.5	0.5
Shortening (g)	5	5	5	5	5
Calcium Propionate (g)	-	-	-	0.5	0.5
Improver (g)	-	-	-	0.5	0.5
Gluten (g)	-	7	12	9	13
Hydrocolloid (g)	-	-	-	0.5	0.5
Flaxseed and muskmelon seed (sparkled) (g)	-	-	-	2.5	2.5

2.6 Proximate composition analysis of different flours

The determination of proximate composition of different flours and bread flour samples was analyzed by moisture content, ash content, protein content, fat content, fiber content. Moisture and ash were determined according to AACC (2000) [11]. Total Dietary Fiber & protein was determined by AOAC [12]. Fat content was estimated by using solvent Petroleum ether. Mineral content was also analyzed using ICP-OES.

Total carbohydrates and energy content were calculated using formulae:-

Total carbohydrate (% dry weight) = {100-moisture (%) - protein content (% dry weight) - crude fat (% dry weight) - ash (% dry weight)} [13]

The calorific value per 100g was calculated according to the system of Atwater (in kcal) using the formula: - Energy (kcal) = (3.36 × % protein) + (3.60 × % carbohydrate) + (8.37 × % fat) [13]

2.7 Preparation of Composite flours extracts for phytochemical analysis

Solvent extraction with ethanol was performed. Fifty grams of composite flours were dissolved in 100ml solvent (ethanol). Then the mixture was incubated in the incubator shaker at 60 °C with 140 rpm for 48 hours. The mixture was filtered through what man filter paper 1 and the filtrate obtained was evaporated, concentrated at room temperature and stored at 4 °C for further use. The yield of all extracts was also calculated. Both, the final composite flours and bread prepared from them were extracted.

2.8 Phytochemical analysis

Total phenolic content of the extract were estimated calorimetrically by Folin-Ciocalteu (FC) method [14]. The absorbance of the standard (gallic acid) and the respective extracts was measured spectrophotometrically at 765 nm against DMSO blank. The results were expressed as gallic acid equivalents (GAE, µg/mg of weight of extract). Total flavonoids were measured by using aluminum chloride colorimetric method [15] and expressed in terms of µg catechin equivalents (CE)/mg of dry extract. The absorbance was taken at 570nm using UV/VIS spectrophotometer. Blank used was DMSO and the same procedure was followed for standard. All the measurements were taken in triplicate. Concentration of crude alkaloids and saponins was also estimated using the methods described by Harborne [16] and Obadoni & Ochuko [17] respectively. The results were calculated in percentage.

Tannins were analyzed according to the method described by Van-Buren and Robinson, 1981 [18] with some modification. Ten grams of the sample was dissolved in 500ml of distilled water. The solution was incubated on shaker for 60 min at 40rpm at 30 °C. Then solution was filtered and 5ml of filterate was taken in test tube followed by addition of 3ml 0.1M $FeCl_3$ in 0.1N HCl and 0.008M potassium ferrocyanide. Take absorbance at 605 nm within 10 minutes. Ascorbic acid was used as standard.

2.9 Antioxidant activity

The antioxidant potential of phenolic compounds was measured by assessing their ability to reduce compounds by

donating electrons using the FRAP assay and DPPH free radical scavenging activity.

2.9.1 DPPH free radical-scavenging activity (2, 2-Diphenyl-1-Picrylhydrazyl)

DPPH scavenging activity was determined by the method of Blois 2000 [19] with slight modification**.** Initially DPPH solution was prepared of 0.3mM concentration by dissolving 1.1829mg in 10ml methanol. Than 3ml mixture was prepared using 1ml of DPPH solution (0.3mM), 1ml of extract (different conc. dissolved in methanol) and 1ml of methanol. The mixed solution was kept in the dark for 10min. The absorbance was measured at 517nm. Methanol was used as a reference and all reagents (2ml methanol+1ml DPPH) used as blank. The % inhibition was determined by:

% Inhibition = ([B-A] /B) × 100

Where: - B= OD of Blank; A= OD of Sample

Ascorbic acid was used as standard.

2.9.2 FRAP (ferric reducing antioxidant power)-

The assay was based upon the methodology of Benzie and Strain [20]. The FRAP reagent was prepared using 10 mM TPTZ in 40 mM HCl, 250 mM sodium acetate buffer (pH 3.6) and 20 mM $FeCl_3$. FRAP reagent was freshly prepared by mixing TPTZ solution, $FeCl_3$ solution and acetate buffer in a ratio 1:1:10. An extract solution (100 µl) was mixed with 900 µl of FRAP reagent. After that the mixture was allowed to stand at 37 °C for 4 min, the absorbance at 593 nm was estimated against the blank. BHT was used as standard. The results were expressed as µg BHT equivalent/mg sample.

2.10 Yield of the extracts

The yield of ethanol extract was calculated using the following formula:

Yield (%) = (weight of the extract obtained/total sample taken for extraction) × 100

2.11 Qualitative analysis of the extracts to screen the presence of phytochemicals

The ethanolic extracts of RWI and RWII were tested for the presence of other phytochemical compounds like terpenoids (Harbrone (1973) [21], anthraquinone (Trease and Evans (1978) [22], Coumarins (Gopinath *et al.*, 2011) [23], Phlobatannins (Edeoga *et al.*, 2005) [24] and Phytosterols (Harbrone (1973) [21].

2.12 Physiochemical Tests

2.12.1 Gluten content-

Gluten content of flour was estimated by following the method No. 38-10 as described in AACC, 2000 [25]. Gluten in a sample of flour can be estimated by washing the dough free of starchy sugar, water soluble proteins and other minor components. The wet cohesive mass obtained is referred to as wet gluten while the dried product obtained from it is referred to as dry gluten. Exactly 2.5g of flour was kneaded with about 15ml of water to get dough ball. Dough ball was allowed to immerse in water for one hour to ensure proper hydration. The starch was washed out by kneading in a gentle stream of water over a fine sieve till washed water passing through the sieve does not turn blue when a drop of iodine solution was added to it. The wet gluten was pressed as dry as possible and weighed. Then placed the wet gluten obtained in dish and dried in hot air oven at 130-133 ℃ for 2 hours. Cool the dried gluten in desiccators and weighed again to get the value for dry gluten.

$$\text{Wet gluten \%} = \frac{A}{C} \times 100$$

$$\text{Dry gluten \%} = \frac{B}{C} \times 100$$

Where, A= wt. of wet gluten (wt. of dish with wet gluten-empty dish wt)

B= wt. of dry gluten (wt. of dish with dry gluten-empty dish wt)

C= wt. of flour sample.

2.12.2 SDS- Sedimentation value

The sedimentation test provides information on the protein quantity and quality of the ground flour sample. The sedimentation value was determined by AACC, 2000 method no. 56-61-3 [25] with a slight modification. Three grams of flour were taken in a 100-milliliter glass-stopper graduated cylinder. 50ml of water was added to the cylinder and mixed for 5 minutes. A 25ml lactic acid solution was added to the cylinder and again mixed for 5 minutes. Then the cylinder was removed from the mixer and allowed to stand in upright position for 5 minutes to sediment the contents. After 5 minutes the volume of sediment was recorded as an SDS sedimentation value in ml.

2.12.3 Particle size index (PSI)

The particle size index of each sample was determined by sieving through sieve shaker model using the method no. 55-30 as described in AACC 2000 [25]. 100 g of flour was weighed and poured over the top of the sieve which is about 412 microns and set sieving balls in every sieve. The weighing pan was set at the end of 180 micron sieve. Weighing pan was cleaned thoroughly before fixing under the sieve. After fixing sieve over the weighing pan, the sieve shaker was run for ten minutes. Five minutes were given to settle the thrush after completion of shaking time. The material passed through the 180 sieve was collected from the pan and measured the passed sample weight through 180 micron sieve.

Percentage of sieved flour through 180 micron:-

$$= \frac{\text{Weight of sieved flour}}{\text{Sample weight}} \times 100$$

2.12.4 Determination of pH

Measurement of pH indicates the acidity/alkalinity in the food sample. The glass electrode pH meter is widely used. Ten grams of the sample was weighed and 40 ml of distilled water was added to the sample. Transfer it to 100 ml volumetric flask and make up to 100 ml with distilled water. pH was determined by dipping the electrode into the solution and allows stabilizing. The pH reading was recorded.

2.12.5 Alcoholic acidity of flour

Alcoholic acidity is defined as mg of NaOH required for 100 g of the sample to have the same alcohol soluble acids. Five grams of sample was weighed and makeup 50ml with ethyl alcohol (85%). The solution was mixed properly and shaken for 1 hour. The solution was filtered and alcohol extract was collected. 10 ml of alcohol extract was taken and added with a few drops of phenolphthalein indicator. The solution was titrated with 0.1 N NaOH solution till light pink color observed. Used NaOH in titration was noted [26].

$$\text{Alcoholic acidity} = \frac{24.52\times V\times N}{W}$$

Where the V = volume of NaOH used in the titration
N = normality of NaOH
W = wt. of the sample.

2.12.6 Yeast Activity

2.12.6.1 Dough Raising Capacity [27] **(**Hamad and Al Eid, 2005 with slight modification)

Yeast both dry and compressed differs in their ability to produce carbon-dioxide in dough because of differences in their activities. It determines suitability of yeast for satisfactory fermentation. 2.5 g of yeast was dissolved in 45 ml of water having 40℃ temperature. 35 g of flour and 1 g sugar was mixed with the yeast suspension in a beaker. This mass was made into a smooth batter and transferred to a 250 ml graduated cylinder and base volume of the batter was noted down. The rise in level of dough was noted after 1 hour.

$$\text{Dough Raising Capacity} = \frac{B-A}{A}\times 100$$

Where, A= volume of the dough before fermentation
B= volume of the dough after one hour fermentation

2.12.6.2 Pop Test

The glass beaker was filled with 10% sugar solution at 32 ℃. 10 g of yeast to be tested was taken and make it into a pellet for dropping it into the beaker. The time taken by the yeast to raise the surface was measured. First pop should come within 10 sec and final pop should come within 60 sec for better result.

2.12.7 Falling Number

For estimating falling number (AACC Approved Method 56-81B) [25] seven grams of properly grounded flour sample were mixed with 25 ml of distilled water in a glass falling number tube with a stirrer and shaken to form slurry. While heating the slurry in boiling water bath at 100 ℃ and stirred constantly, the starch gelatinizes and forms a thick paste. The time taken by stirrer to drop through the paste is recorded as falling number value. Sound wheat takes 200-350 seconds to drop.

2.13 Chromatographic Analysis

2.13.1 GC-MS Profiling

Secondary metabolites present in the sample of composite flour extracts were analyzed by using 1µl each, of ethanol extract of the sample by using GC/MS analysis. Helium was used as carrier gas. An Agilent 6890 GC with 5975B mass spectrometric detector (MSD) was used in the scan mode (m/z 35-1050) for the sample. Screening of volatiles and semi volatiles were performed using the automatic RTL screener software in combination with the Agilent NIST'05 library **(**Pasricha, 2014**)** [28]. The temperature for the analysis was set to 300 °C, solvent delay was 3 min, ion source and quadruple temperature were 230°C and 150 °C, respectively **(**Adams, 1989) [29]. The detected compounds have been identified by the NIST'05 mass spectrum library.

2.13.2 HPLC

High performance liquid chromatography (HPLC) is an important qualitative and quantitative technique, generally used for the estimation of pharmaceutical and biological samples.

2.13.2.1 Apparatus, reagents and HPLC conditions-

The chromatographic system includes an Agilent infinity 1200 series system: - gradient pump, a stainless steel injector (5 µL loop), and a UV-VIS detector and Diode array detector with data acquisition by EZ chrome elite software. The optimum conditions for HPLC were developed for the identification and quantification of desired compounds. The compounds desired to quantify were quercetin, rutin, chlorogenic acid and epicatechin.

Preparation of Test Sample and Reference solution:-

Test solution- 100mg of extracts were dissolved in 5ml of methanol. The prepared solutions were sonicated for 10 minutes and then filtered the extract solution to avoid the clogging of the column. In this research two test samples were taken.

Reference solution- A stock solution of reference solution was prepared by dissolving 1 mg of reference in 5 ml of methanol. This solution was sonicated for 10-15 minutes. Four references were taken: - quercetin, rutin, chlorogenic acid and epicatechin.

The optimum conditions for quantification of quercetin were:- i) Column used-stainless steel column 25cm×4.6mm× 5µm packed with octadecylsilane bonded to porous silica, ii) Flow rate of the mobile phase was -1 ml/min, iii) Mobile phase – a mixture of 40 volumes of acetonitrile and 60 volumes of a buffer solution prepared by dissolving 3.01g of potassium di-hydrogen orthophosphate in 1000ml of water and adjust the pH to 2 with acetic acid, iv) Column temperature was kept at 25 °C, v) The sample solution and reagent solution were degassed before each run, vi) Injection volume-10 µl, vii) Run time- 15 minutes, viii) Spectrophotometer wavelength- 370nm.

The optimum conditions for quantification of chlorogenic acid, epicatechin and rutin were same: - i) Column used-stainless steel column 25cm×4.6mm× 5µm packed with octadecylsilane bonded to porous silica. ii) Flow rate of the mobile phase was -1 ml/min, iii) Mobile phase – 1% aqueous acetic acid solution (A) and methanol (B), iv) Column temperature was kept at 25 °C, v) The sample solution and reagent solution were degassed before each run, vi) Injection volume-10 µl, vii) Run time- 15 minutes, viii) Spectrophotometer wavelength- 278nm.

The reference solution and the test solution were injected. The content of quercetin, epicatechin, rutin and chlorogenic acid were calculated by using a formula:-

$$\text{Area \%} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Standard mg}}{\text{Standard dilution}} \times \frac{\text{Sample dilution}}{\text{Sample mg}} \times \frac{\text{Standard Potency}}{100}$$

2.14 Functional Properties

All composite flour blend and refined wheat flour were tested for functional properties such as bulk density (Okaka and Potter 1977) [30], water holding capacity, water absorption capacity (Gould *et al.* 1989) [31], oil absorption capacity (Sosulski *et al.* 1976) [32], swelling index (Lin *et al.* 1974) [33], foam capacity and foam stability (Narayana and Rao 1982) [34].

2.15 Physical analysis of experimental breads

Loaf height and loaf weight were measured through the graduated scale and calibrated weighing scale respectively.

2.15.1 Physical Baking losses

The baking losses were analyzed in terms of moisture loss (Menon *et al.* 2014) [35]. This was determined by comparing the difference in weight of the dough and the final baked bread. This baking loss is supposed to be due to the loss of moisture of bread during baking and expressed in percentage.

2.16 Organoleptic evaluation

Firstly the bread samples were allowed to cool for 1-2 hours at room temperature then sliced into pieces. The organoleptic evaluation of the breads was determined by sensory panel. The panelists were from the members of the postgraduate food technologist of GGSIP University. A 9-point hedonic scale was used to mark the sensory scores for the breads evaluation. Sensory analysis of the functional composite breads was carried out by 10 panelists on a 9 point hedonic scale for different parameter such as crumb structure, crust color, aroma, taste, mouth feel and overall palatability.

2.17 Antibacterial activity

Antibacterial activities of the extracts were evaluated by an agar well diffusion method against three Gram-positive bacteria and three Gram negative bacterial test pathogens. The ethanolic extracts were reconstituted to final concentrations of 100 mg/ml and 300mg/ml. Two different solvents DMSO and methanol were used to dissolve the ethanolic extracts for performing antibacterial assay. Nutrient agar was inoculated by spreading 100 µl of the bacterial inoculums. 100µl of ethanolic extracts were loaded into the wells (6 mm diameter) prepared. After inoculation the plates were incubated at 37 ºC for 24 h. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition and reported on the scale of millimeters. DMSO and methanol were used as control in one of the wells [28].

3. Results and discussion

3.1 Bread preparations

Four composites flours, which were taken:- 1) 50% refined wheat, 10% chickpea, 10% buckwheat, 10% sorghum, 10% sprouted barley and 10% sprouted wheat (MI) **;** 2) 50% whole wheat, 10% chickpea, 10% buckwheat 10% sorghum, 10% sprouted barley and 10% sprouted wheat (MII); 3) 65% refined wheat, 5% chickpea, 5% buckwheat 5% sorghum, 5% sprouted barley, 5% sprouted wheat, 5% corn flour and 5% defatted soy flour (RWI); 4) 65% whole wheat, 5% chickpea, 5% buckwheat, 5% sorghum, 5% sprouted wheat, 5% sprouted barley, 5% corn flour and 5% defatted soy flour (RWII).

Formulation MI and MII were not taken for further studies because during dough kneading the gluten network was not developing properly and the dough does not get desirable elasticity. Therefore bread prepared from MI and MII composite flours do not provide the desirable crumb structure and overall bread rheology.

To overcoming these rheological problems RWI and RWII formulations were prepared further, where additional flours like corn flour and defatted soy flours were availed to get desirable crumb structure. To increase the gluten content and elasticity, 65% of refined wheat was taken (RWI) with other grains. Hydrocolloid was also added in combination with gluten. To make the results comparable other formulation (RWII) was taken in where 65% of refined wheat was replaced with whole wheat, other grains quantity (35%) was same as of RWI. After baking of dough, bread prepared was giving after taste. According to literature, it may be due to flaxseed. To overcome this problem on next trail the flaxseed used was roasted flaxseeds, which does not impart after taste after baking.

Fig 1: Crumb structure

3.2 Rheological Acceptance

On the basis of rheological acceptability the formulation 3 (RWI) and 4 (RWII) were opted for further experimental analysis. From the above figure1 it can clearly be concluded that formulation 3 and 4 have the desirable crumb structure. BI and BII bread prepared from RWI and RWII flour was further evaluated on the basis of sensory and nutritional.

Different parameters were observed during bread preparation from RWI and RWII flours. The table 2 shows the observations.

Table 2: - Different Bread parameters observed

Parameters	Control	RW Formulation I	RW Formulation II
Mixing time (min)	7	7	11
Dough temp (℃)	27.4	28.5	29.5
Dough weight (gm)	511	561	575
Scaling weight (gm)	460	470	470
No. of Dough Unit	1	1	1
Fermentation time (min)	30	45	50
Baking time (min)	30	47	47
Baking temperature (℃)	210(℃)/200(℃)	210(℃)/200(℃)	210(℃)/200(℃)
Final Loaf weight	405	427	434
Baking loss	55	43	36

RWI RWII BI BII

Proofed dough After baking

Bread prepared from RWI & RWII

Fig 2: Proofed dough and Bread prepared from RWI & RWII

3.3 Organoleptic evaluation

The sensory evaluation and organoleptic properties of experimental breads and control is presented in the figure 3. The evaluation was done on a nine-point hedonic scale. The crust color, aroma, texture, flavour, mouthfeel and overall palatability of all two breads were compared with the control bread. The crust color of the bread was darker in BII bread, when compared to control and BI bread. The more brownish bread appearance could be attributed to the high fiber content in the bread [36]. The brown color of the bread is due to the caramelization and maillard reaction, in which protein and sugar of the flours react with each other during baking process [37].

The texture (softness and chewiness) of the composite bread decreased as the composite flours substitution increases. This characteristic can be attributed to the high fiber content of the bread. The best bread BII according to nutrition, with more substitution with whole multigrain or composite flours had the acceptable texture. All the components of the flour like protein, fiber, gluten, starch, the amount of water required for dough mixing contributes to the final texture of the bread [38].

The flavor and aroma of both the BI & BII breads were good as compared to control bread. Most of the panelists judged the grainy taste of the breads due to the presence of whole multigrains. Roasted flax seeds and muskmelon seed impart better flavor and aroma to the bread.

The sensory evaluation of the breads revealed that both BI & BII bread were acceptable, but on nutritional point of view bread (BII) which was prepared from 100% whole grains was considered more healthy (65% whole wheat, 5% chickpea, 5% buckwheat, 5% sorghum, 5% sprouted wheat, 5% sprouted barley, 5% corn flour and 5% defatted soy flour). Further analysis was done on both the breads and their respective composite flours.

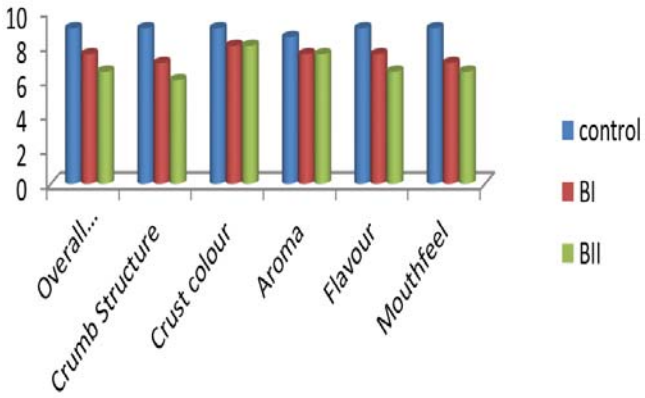


Fig 3: Sensory evaluation of formulated Breads (BI &BII)

3.4 Proximate analysis of the different composition flour and bread

Proximate analysis is important for evaluating the nutritional content of the developed food products. The different chemical composition of composite flour affects the nutritional quality of the product. The total protein, dietary fiber, moisture, fat, ash, carbohydrate and energy content in composite flours and final bread prepared from them are shown Table 3.

Besides providing essential nutrition, protein can also be related to finished product attributes like texture and appearance, processing properties such as water absorption and dough development time because high protein in flour usually requires long mixing time and more water to achieve dough consistency while low protein content require low water to develop the dough. The protein content of experimental composite flours i.e. RWI and RWII were- 11.57% and 12.10

respectively, which were significantly higher than refined wheat flour which is about 11%. This significant increase in the protein content in the composite flours could be attributed to high protein content in individual flours. The crude fat content of RWI and RWII flour were 1% and 1.32%, respectively, while the fat content of the refined flour was 0.9%. The high fat content in composite flours could have the ability to make bread without or less addition of shortening agent. The main role of fiber is to keep the digestive system healthy. Fiber has also been shown to benefit diabetes [39], blood cholesterol levels [40], reduces constipation, coronary heart disease [41], and obesity [42]. On the other hand fiber has negative impact on dough rheology. It reduces product volume, texture and the ability of gluten protein to aggregates during the making of the dough. More fiber content in the flour will take more time for dough mixing. The fiber content of the RWI & RWII is shown in the table below. Grain quality is very much depends on its moisture content. Moisture content is the indicator of grain storability. High moisture content increases the microbial activity which deteriorates the product during storage. Moisture content more than 14% is not acceptable. The ash is composed of non-combustible, inorganic minerals that are concentrated in the bran layer. Ash content can be attributed to the mineral content in the sample.

Table 3: Nutritional profile of RWI, RWII and control (*control result is referred from nutritive fact data)

Sample	Energy (Kcal/100g)	Total Carbohydrate%	Protein%	Fat%	Dietary Fiber%	Moisture%	Ash%
Control flour	348	73.9	11	0.9	2.4	10.2	0.58
RWI flour	313.03	73.83	11.57	1	12.61	12.5	1.1
RWII flour	316.16	73.46	12.10	1.32	14.82	11.7	1.42

Table 4: Extract yields of raw formulations and bread prepared

Samples	Solvent used for extraction	Yield %
RW I	Ethanol	1.62
RW II	Ethanol	2.18
Bread I	Ethanol	5.9
Bread II	Ethanol	5.8

Comparative analysis

Table 5: Comparative analysis of control (refined and whole wheat bread) with experimental bread (BII* nutritionally rich and organoleptically accepted)

Sample	Energy (Kcal/100g)	Total Carbohydrate%	Protein%	Fat%	Dietary Fiber%
Control bread	217-266	47.38-56.94	5.9-7.96	3	0.25-0.30
Whole wheat bread	217-240	48.90 –54.51	8.31–9.20	1.6	0.87–0.93
BII bread	219.91	38.12	18.83	2.32%	8.39

The mineral content in composite flour RWII was analyzed by Inductively coupled plasma atomic emission spectroscopy. On the nutritive and a sensory basis RWII flour was considered best for ICP-OES. Major mineral is Phosphorous which include many health benefits such as bone formation, proper digestion, regulated excretion, formation of protein, hormonal balance, improved energy extraction, cellular repair and also helps in constipation, diarrhea, healthy bowl movements and also helps in the treatment of cancer and calcium which helps in strengthening bones and teeth, may combat cancer.

Table 6:- Mineral content of MIV flour by ICP-OES

Minerals (ppm)	MIV flour (ppm)
Ca	280.50
Cu	2.5
Cr	1
Fe	58.75
Mg	560
Mn	12.5
P	18225
Sr	2.5
Zn	12.5

3.5 Phytochemical Analysis

Phytochemical analysis showed the presence of phenolics, antioxidant, alkaloids, saponins and flavonoids and slight amount of tannins in both raw RWI, RWII extracts as well as in bread I and II extracts. Phenolics and flavonoids are natural compounds found in many cereal grains. A review published in 2012 found growing consensus for the hypothesis that the specific intake of food and drink containing relatively high concentrations of flavonoids may play a meaningful role in reducing the risk of cardiovascular disease (CVD). Phenolics have many health benefits like antioxidantive, antiinflammatory, antimutagenic and anticarcinogenic properties. It also maintains flavor, taste, color and prevention of their oxidation deterioration. The total phenolic content of the extracts of RWI, RWII and Bread I and Bread II extract are shown in Table 7. The results were expressed as respective standard equivalents (µg/mg of dry weight of extract).

Tannin, an astringent plant polyphenolic compound, was present in slight amount in both the composite flours extracts, which is shown in Table 7. Crude alkaloids in the RWI and RWII extracts were estimated to be 0.46% and 0.365% respectively. Their presence in moderate amount divulges its pharmaceuticals and therapeutic significance. Saponins are a class of chemical compound found in many plant species especially in legume plants [43]. It poses a wide range of bioactive activities.

Table 7: Phytochemicals present in RWI, RWII, BI & BII extracts. * Calculated for powder

Analyte	Total Phenolics (µg GAE/ mg sample)	Total Flavonoids (µg QE/ mg sample)	Crude Alkaloid Content (%) *	Tannin*	Saponins Content (%)*
RWI extract	11.8	51.2	0.46	0.159	6.5
RWII extract	15.8	48	0.365	0.003	8
BI extract	15.75	21.6	0.295	0.162	7.5
BII extract	17.87	106.4	0.775	0.068	8

3.6 Antioxidant activity analysis

Phenolic compounds can also act as antioxidants by reacting with and capture dangerously reactive compounds called free radicals before the radicals can react with other biomolecules and cause serious damage. Total antioxidant is measured in terms of radical scavenging activity of antioxidant agents free radicals like DDPH radical and by FRAP assay. The primary sources of naturally occurring antioxidants are whole grains, fruits & vegetables. DPPH is widely used free radical to test the ability of compounds to act as free radical scavengers & to evaluate the antioxidant activity of foods. DPPH is a stable free radical with violet colour. If free radicals have been scavenged, DPPH will change its colour from violet to pale yellow or colourless. The result revealed that the ethanolic extract of composite flours exhibited the good antioxidant and free radical inhibiting activity at 1500PPM. This can be attributed to the presence of multigrains mix taken in the study.

Table 8: BHT equivalents (µg BE/mg sample) and DPPH activity (% Inhibition) of composite extracts.

Extracts	RWI	RWII	BI	BII
FRAP(µg BE/mg extract)	815.2	466	627.68	375.38
DPPH (% I) In 1500ppm extract	24.59%	32.37%	21.38%	36.45%

3.7 Comparative result analysis of raw flour and bread extracts

It can be observed from the table 7 that there is an increase in the phenolic and flavonoid content of flours after baking. Some flavonoids, compounds decrease during baking as in case of BI. Thermal processes have a large influence in flavonoid availability in food which depends on their magnitude and duration. Different studies have been done on effect of different heating methods on flavonoids and phenolic. Studies reveal that in some cases, an increase of temperature improves the extraction of phenolic compounds from foods; others results showed losses of phenolic compounds in different quantities [44]. The effect of different type of heating method was also studied (roasting 20 min -40 min at 80 degrees and 120 degrees, pressurized, steam-heating (0.1 MPa, 20 min; 0.2 MPa, 40 min), microwaving (700W, 10 min)), on buckwheat, which shows 20-30% increases in flavonoid content [45]. Different studies show increases in different individual phenolic compounds after heating. By present study, it can also be concluded that high baking temperature (200 ℃-210 ℃) increases the flavonoids and phenolic content in bread when compared with initial raw flour. It is not necessary that with the decrease of phenol content antioxidant activity decreases. It was revealed in study that the degradation products of phenolic compounds can also have antioxidant activity higher than the initial phenolic compounds [46, 47]. Therefore, it cannot be concluded that baking or high temperature would always decrease the phytochemical compounds. It depends on the interaction of the food matrix and compounds.

3.8 Qualitative analysis of the extracts

Table 9: Qualitative analysis of the extracts

Herbal extracts	Phytosterol	Terpenoids	Phlobatannins	Anthraquinone	Coumarin
RW I	+	+	_	_	+
RW II	+	+	_	_	+

Ethanolic extracts were also subjected for qualitative tests such as phytosterol, terpenoids phlobatannins and anthraquinone, coumarin. Qualitative tests show the positive results for phytosterol, terpenoids and coumarin (Table 9). Terpenoids are the largest groups of phytochemicals and used as a chemopreventive and therapeutic agents in liver cancer. They also have antioxidant activity [21]. Coumarin has been shown to activate other cells of the immune system. It also prevents from leukemia. This compound was known to inhibit tumor spread, and to stimulate granulocytes, lymphocytes and macrophages [48]. Coumarins can be used not only to treat cancer but to treat the side effects caused by radiotherapy. Phytosterols are plant (Phyto) chemicals. The three most common forms of phytosterols in foods are beta-sitosterol, campesterol, and stigmasterol. It may offer protection against cancer by several different means. These include inhibiting cell division, stimulating tumor cell death, reduces cardiovascular disease [49]. Phytosterols also help in lowering cholesterol in the body.

3.9 Physiochemical Analysis

The gluten content helps in analysis the quality of the wheat flour, which can be used for different products as per the specification required. Sedimentation value test provides information on the protein quality and quantity of ground wheat and flour samples. Positive correlations were observed between sedimentation volume and gluten strength or loaf volume attributes. It is used as a screening tool in wheat

breeding as well as in milling applications. According to predefined specifications the formulated flours comes under medium wheat flour category. Particle size index (PSI) indicates the structure of grains. The more intact the structure of a cereal grain, the more slowly the starch within will be digested. Larger food particles have a lower surface-to-volume ratio and this may reduce the excess of enzymes to the interior of the particle. PSI also influences the package design and material required for packaging.

Alcoholic acidity determines the age of the flour and shelf life of the bread. Grains or their milled products in storage undergo physical as well as chemical changes. Acid phosphatase, amino acids and free fatty acids of flours, under certain conditions increase due to the enzymatic hydrolysis of proteins and fat, respectively. The amino acids, acid phosphates and free fatty acids are soluble in strong alcohol. It is expected to be low, and 'lower the better' in bread. The alcoholic acidity should not come more than 0.1%. According to the specifications the pH of the bread should lie between the 5-6. pH determination is an important measure of the active acidity, which influences the flavor or palatability of a product and affects the processing requirements.

Table 10: Physiochemical value of raw flour formulations

Test sample	SDS value (ml)	Gluten content	PSI %	Alcoholic Acidity	pH of respective breads
RWI	24	11.96%	98	0.14	5.71
RWII	17	10.10%	98	0.18	5.60

Yeast Activity is estimated by dough raising capacity and pop test which determines the suitability of yeast for a satisfactory fermentation. The first pop should come within 10 second and final pop should come within 60 seconds for suitable yeast. Compressed yeast taken in the present research shows first pop within 20 second and final pop was within 80 seconds. Falling number is an important test to be conducted in the bakery industry before using any flour. It measures the level of enzyme activity which affects the product quality. If the falling number is too high, enzyme can be added to flour to compensate. If the falling number is too low, enzyme cannot be removed from the flour. Too much enzyme activity means too much sugar and too little starch. More enzymes results in sticky dough during processing and poor texture of finished product. Sound grain gives falling number value between 200-350 seconds and the rain infected grain value gives below 200 seconds.

Table 11: Falling Number of raw flour formulations

Test sample	Falling Number
RWI	297
RWII	302

3.10 Chromatographic Analysis

3.10.1 Characterization of GC-MS analysis

The GC-MS analysis led to the identification of common fatty acids and other phytochemicals from a GC fraction of ethanolic extract of RWI & RWII sample. The identified secondary metabolites of composite flour extracts (RWI and RWII), their retention indices, percentage composition and activities are given in Table 12 and 13 below.

From the result, it was observed that the ethanolic extracts of RWI & RWII showed 9 and 15 compounds respectively. In terms of percentage amounts Octadecanoic acid (16.02%) & n-Hexadecanoic acid (14.18%) were predominant in the RWI and RWII extracts respectively. These major compounds have shown to have many health beneficial activities. The presence of so many antioxidants compounds in composite flour like Pentadecanoic acid; Hexadecanoic acid, methyl ester; 2,6,10,14,18,22 tetracosahexaene,2,6,10,15,19,23-hexamethyl-, (all-E); beta tocopherol, vitamin E justifies the high antioxidant properties. CAS Number in the table indicates unique numerical identifier assigned by the Chemical Abstracts Service (CAS) to every chemical substance described in the open scientific literature.

Table 12: Secondary metabolites in GC-MS analysis of RWI flour extract

Compound name	RT	% Area	CAS#	Biological Activity
Tetradecanoic acid	18.149	0.71	000544-63-8	Antioxidant, cancer preventive, nematicide, hypercholesterolemic, Lubricant, flavoring agent
Pentadecanoic acid	19.192	0.98	001002-84-2	Antioxidant
n-Hexadecanoic acid	20.246	11.69	000057-10-3	Antioxidant, hypocholesterolemic, nematicide, hemolytic, 5-alpha reductase Inhibitor
Octadecanoic acid, methyl ester	21.715	0.37	000112-61-8	anti-inflammatory, cancer preventive, hypocholestrolemic, antiarthritic activity.
9-Octadecenoic acid, (E)-	21.929	10.17	000112-79-8	Antioxidant, nematicide, cancer preventive, hypocholestrolemic, antiarthritic activity.
Octadecanoic acid	22.108	16.02	000057-11-4	Antioxidant, anti-inflammatory nematicide, cancer preventive, hypocholestrolemic, antiarthritic activity.
Stigmastan-3,5-diene	31.035	1.99	1000214-16-4	Dehydratation of b-sitosterol yields stigmasta-3,5-diene(M. León-Camacho *et al.*, 2004).
beta.-Sitosterol	35.275	4.37	000083-46-5	treatment for high cholesterol, enlarged prostate & enhance immune function
Heptadecanoic acid	21.166	0.68	000506-12-7	Antioxidant

Table 13: Secondary metabolites in GC-MS analysis of RWII flour extract

Compound name	RT	% Area	CAS#	Biological Activity
2-Methoxy-4-vinylphenol	12.687	0.09	007786-61-0	flavoring agent, responsible for the natural aroma of buckwheat (Janes & Kantar *et al.*, 2008). anti-inflammatory effects.
Tetradecanoic acid	18.160	0.20	000544-63-8	Antioxidant, cancer preventive, nematicide, hypercholesterolemic, Lubricant, flavoring agent
Trichloroacetic acid, tetradecyl ester	18.418	0.25	074339-52-9	To treat skin local lesions and various dermatological diseases
Pentadecanoic acid	19.203	0.15	001002-84-2	Antioxidant
Hexadecanoic acid, methyl ester	19.798	0.32	000112-39-0	Antioxidant
n-Hexadecanoic acid	20.415	14.18	000057-10-3	Antioxidant,hypocholesterolemic, nematicide, hemolytic, 5-alpha reductase inhibitor
8,11-Octadecadienoic acid, methyl ester	21.480	1.44	056599-58-7	anti-inflammatory, cancer preventive, hypocholestrolemic, antiarthritic activity.
9,12-Octadecadienoic acid (Z,Z)-	22.231	49.51	000060-33-3	Antioxidant, cancer preventive,
9,12-Octadecadienoic acid (Z,Z)- 2-hydroxy-1-(hydroxymethyl)ethyl Ester	26.448	5.85	003443-82-1	Antioxidant, cancer preventive,
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all- E)-	27.536	1.30	000111-02-4	Antioxidant
Eicosane	28.164	1.36	000112-95-8	antitumour activity
beta.-Tocopherol	30.284	0.67	000148-03-8	protection from the nitrogenoxide, a free radiclal and from inflammation
Vitamin E	31.540	0.50	000059-02-9	treatment & prevention from heart diseases, diabetes and lung cancers, aging, inflammation.
5-Cholestene-3-ol, 24-methyl-	33.346	1.04	1000214-17-4	controls cholesterol and lowering the risk of heart diseases
Stigmasterol	35.297	1.23	1000214-20-7	anti-diabetic and thyroid inhibiting properties

3.10.2 HPLC Analysis

HPLC is one of the best separation techniques of column chromatography for the quantification of different phytochemicals present in a sample. In present study four phytochemicals i.e. quercetin, epicatechin, rutin and chlorogenic acid were quantified by HPLC. The standards and sample retention time and % area are given in Table 14. HPLC method was developed for the quantitative estimation of all four phytochemicals from ethanolic extracts. Stainless steel column 25cm x 4.6mm which was packed with octadecylsilane bonded to porous silica (5µm) as a stationary phase was used and different mobile phase (as discussed in section 2.13.2.1) was used for analysis of phytochemicals in extracts.

The calculation for quantifying quercetin, epicatechin, rutin and chlorogenic acid in the different extract sample by using formula:-

$$\text{Area \%} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Standard mg}}{\text{Standard dilution}} \times \frac{\text{Sample dilution}}{\text{Sample mg}} \times \frac{\text{Standard Potency}}{100}$$

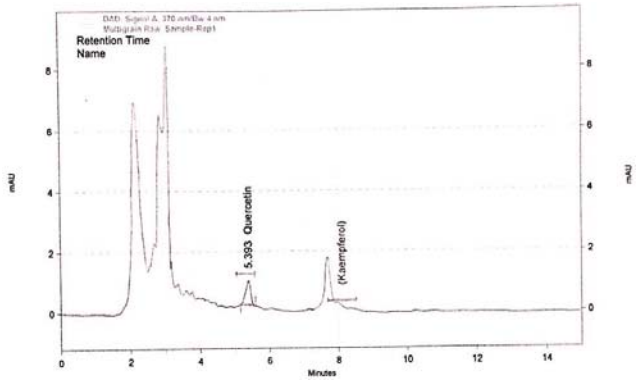
The HPLC analysis of RWII flour and BI was done. All four standards (quercetin, epicatechin, rutin and chlorogenic acid) were run for both the samples. Raw formulation 1 shows the presence of quercetin, epicatechin and chlorogenic acid. Rutin was present below the LOD level. Fig.4 shows the chromatogram of raw formulation 1.

In bread formulation 2 (BII) the amount of chlorogenic acid and rutin was increased, but the quercetin amount is decreased and epicatechin was degraded completely. The reason behind the decreasing amount of quercetin could be due to the effect of baking temperature on quercetin. The processing techniques may have an impact on flavonoids structures which may result in changes in the activity and bioavailability of flavonoids. Quercetin glycoside under roasting condition (180 °C) degrades which was previously studied [50]. Also it was observed that the quantity of epicatechin was degraded completely during baking condition (210 °C) because that Catechin and epicatechin content decreased with increasing heating temperature (≥180 °C), according to research done on grape seed polyphenolic content [51].

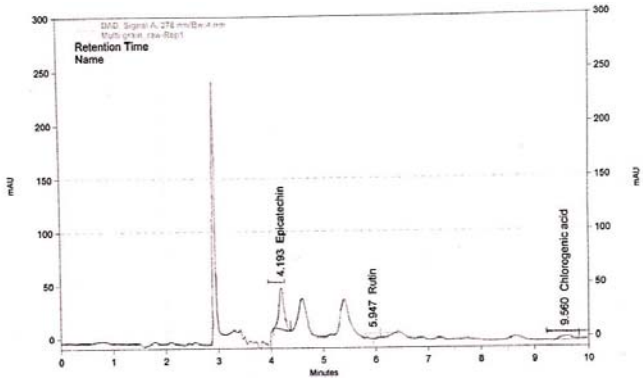
There was a slight increase in rutin quantity was observed in bread sample. It could be due to the different nature of the different glycosides present. The stability of the glycosides is dependent on the kind and the position of the sugar moiety [50]. It is not necessary that glycosides would decrease in baking temperature, other structural changes may occur. But the quantity of chlorogenic acid was increased in high amount after baking. It is possible that other chemical transformations may have occurred at high temperature or small molecule fragments could have linked together and form chlorogenic acid. The high temperature of the roasting process causes a breakage of the carbon-carbon bonds of chlorogenic acid (CGA), resulting in isomerization and degradation. After 5 min of roasting (7% weight loss), the levels of 5-Caffeoylquinic acid (5-CQA) had decreased substantially, while the levels of 3-CQA and 4-CQA had increased to twice their original values [52].

Table 14: The Retention time of quercetin, epicatechin, rutin and chlorogenic acid present in the raw flour formulation II and bread formulation II, detected by HPLC.

Quantified Compounds	Raw Formulation 2		Bread Formulation 2	
	% Area	Retension time	% Area	Retension time
Quercetin	0.115518	5.39	0.108292	5.58
Rutin	0.000949	5.94	0.012511	6.13
Epicatechin	0.132867	4.18	Degraded	-
Chlorogenic acid	0.580150	9.55	2.199488	9.53



(a)



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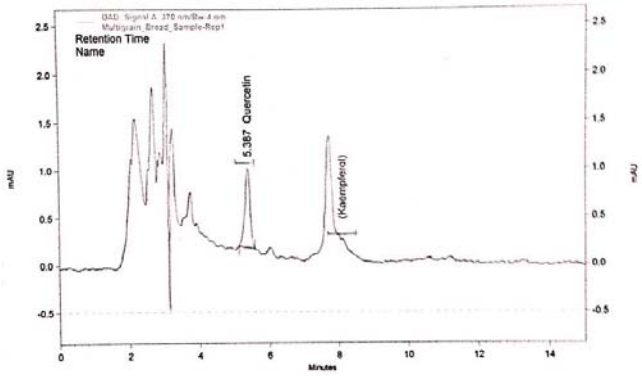

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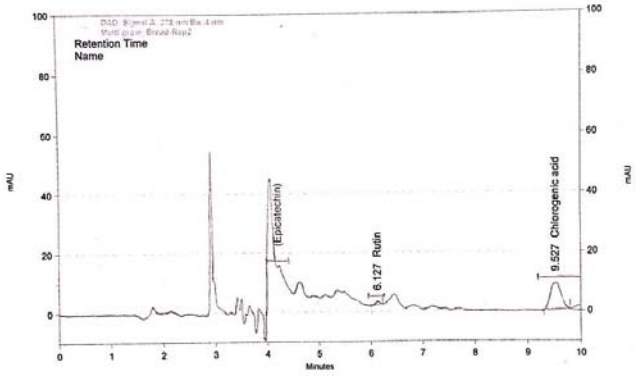

(d)

Fig 4: HPLC separation of quercetin in RWII & BII (a, c) and epicatechin, rutin chlorogenic acid in RWII & BII (b, d)

Table 15: Health benefits of quantified compounds from the extracts of raw formulation II & bread formulation II

Quantified Compounds	Health Benefits
Quercetin (Flavonols)	It inhibits enzyme aldose reductase, which plays a role in converting glucose (sugar) to sorbitol (a sugar alcohol) in the body, nutritional management of diabetes. Prevents cancer, have Anti-oxidant property, protects against coronary heart disease (CHD), prevention of peptic ulcer, protect the brain functions [53]
Rutin (Flavonols)	Have antioxidant property, prevent diabetes, anti-inflammatory, anti-carcinogenic properties, stroke prevention decreased the levels of LDL cholesterol [54]
Epicatechin (Flavanols)	Prevent blood pressure increase & diabetes, strong antioxidant, prevents from the risk of CVD, Prostate Cancer, lung cancer [55]
Chlorogenic acid (guinic acid)	Have antioxidant properties, reduction of the relative risk of cardiovascular disease, diabetes type 2 and Alzheimer's disease [56, 57]

Bulk density is not considered an intrinsic property of a material; it can change depending on how the material is handled. The higher bulk density in individual and composite flours is attributed to the high fiber content in the flours [58]. Bulk density generally varies with respect to variation in particle size.

3.11 Functional properties of composite flours

Functional properties are the intrinsic physiochemical characteristics which may affect the behavior of food systems during processing and storage [35].These properties will decide the acceptability of the product. The water holding capacity (WHC) is the first functional properties to determine the types of flours to use. RWI & RWII flours show higher WHC and Water absorption capacity (WAC) than the refined wheat flour, which can be attributed to high fiber and starch content in the flour. More amount of water is needed for multigrain dough formation than in the refined wheat dough formation. Oil absorption capacity (OAC) of control refined wheat flour (RWF) was more than composite flours. OAC of protein depends upon the intrinsic factors like amino acid composition, protein conformation. The OAC also affects the flours flavor and mouth feel [59]. The increase of oil absorption may be attributed to the presence of more hydrophobic groups or polar amino acid on the surface of protein tends to decrease oil absorption capacity [60]. Therefore the lower oil absorption capacity of composite flour (RWI & RWII) might be due to the presence of high protein of hydrophilic nature, like in legume flour.

High protein and fat content reduce the swelling index of the flours which inhibits the process of starch gelatinization. Protein and starch form a complex which results in reduction of swelling index of the flours [61]. Swelling index also affects the final loaf volume of bread. Foaming capacity of refined wheat flour was higher than all other flours. Foaming capacity depends on configuration of protein molecules. Flexible proteins have good foaming capacity, but highly ordered globular molecule gives low foam ability [62]. Thus the reduction in foaming capacity for composite flour blends in the present study indicates the ordered globular nature of the proteins contained in it.

Table 16: Estimation of functional properties of composite flours

Parameters	Control flour	RWI	RWII
Bulk density (g/ml)	0.923	0.925	0.934
Water Holding Capacity (g/g)	2.04	2.18	2.6
Water Absorption Capacity (%)	55	58.4	58
Oil Holding Capacity(g/g)	2.5	2.0	1.8
Swelling Index	3.5	1.75	1.32
Foaming Capacity (ml)	4	1.6	2.4

3.12 Physical analysis of experimental breads

The loaf weight, loaf height and baking losses were also evaluated. Loaf weight is determined by the amount of moisture and carbon dioxide diffuse out of the dough during baking. More loaf weight could be attributing to protein and fiber rich grain incorporated and carbon dioxide diffused. Loaf weight of standard refined wheat bread was less than the experimental BI and BII breads. Dough also losses weight by a small amount of dough that sticks to the mixer. This amount of loss averages, about 2 percent. Dough also loses weight during the proofing, baking and cooling (11%).

Loaf height of the BII bread was less than the control bread and BI. The presence of more fibrous flour causes a subsequent reduction in loaf volume. In control and BI bread more proportion of refined wheat was present, thus gluten formation was more which might help in the retention of more carbon dioxide gas resulting in higher loaf height. There is a negative influence of the fiber on bread volume, which is related to reduce the ability of the gluten to aggregate and affects the products volume and texture. It also reduces crust crispness.

Baking loss can be analyzed in terms of moisture loss. The moisture content of the control bread was less than the composite flour breads. During baking, the loss of moisture content decreases with an increased proportion of composite flour and thus baking loss also decreases of composite flour bread. Since composite flours have more water holding capacity therefore has more moisture content [35].

Table 17: Physical attributes of control and experimental breads.

Parameters	Control flour	BI bread	BII bread
Loaf weight (g)	405	427	434
Loaf Height (cm)	11.5	11	10
IPT (℃)	90	91.6	91.8
Baking loss %	55	43	36

3.13 Antimicrobial test

Six test pathogens were taken for evaluating antimicrobial activity of raw formulation II (RWII). The bacterial strains used in this study were *S. aureus, S. epidermidis, B.cereus* (gram positive) and *B. subtilis, S.enterica, E. coli* (Gram negative). Agar well diffusion method was used for assessing the antibacterial activity of RWII sample against test pathogens. The zones of inhibition were obtained after 24 hours. Inhibiting concentration used in the sample extract were 100mg/ml and 300mg/ml, dissolved in DMSO and methanol. The ethanolic extract of the formulation (RWII) of composite flour showed maximum antibacterial activity against *S. enteric* (Gram-negative), *S. aureus* (Gram-positive), and showed low antibacterial activity against *S. epidermidis* (Gram-positive). The extracts, dissolved in DMSO were most active against the Gram-negative against bacteria (*S. enteric*), tested at two different concentrations.

Table 18: Antibacterial activity against various bacteria for Composite flour formulation I and II

Test pathogens	Composite raw formulation II zone of inhibition (mm)					
Gram positive	**DMSO (100mg)**	**Methanol (100mg)**	**DMSO (300mg)**	**Methanol (300mg)**	**DMSO control**	**Methanol Control**
S. aureus	10	14	11	12	-	-
S. epidermidis	-	9	-	10	-	10
B.cereus	8	15	9	9	-	-
Gram negative						
B. subtilis	10	10	-	-	-	-
S. enterica	20	10	23	20	-	-
E. coli	-	15	-	11	-	-

4. Conclusion

It can be concluded from the present study that use of the formulated composite flour can be considered in the preparation of the bread. On nutritive profiling of the composite flour they showed high amounts of protein, fiber, low fat, moderate carbohydrate and high mineral content. Phytochemical analysis of RWI and RWII flour extracts also shows a good amount of flavonoids, phenolics, saponins and antioxidant properties. Composites flour prepared using whole grain flours of sorghum, chickpea and buckwheat, sprouted barley and sprouted wheat blended with refined wheat flour and whole wheat flour, not only increases the nutritive value but also the phytochemical characteristic of the bread. From the study it was revealed that nutritionally and organoleptically accepted bread (BII) after baking shows high flavonoid content 106.4 µg QE/ mg sample, the moderate phenolic content with 17.87 µg GAE/ mg sample and good antioxidant activity with 36.45% inhibition and 375.38 µg CE/mg. Bread prepared from these composite flours (RW I and RW II) showed that, the presence of multigrain flours in lower proportion yielded bread (B-I) with better organoleptic and physical properties when compared with control refined wheat bread. The composite bread (B-II) also consist high amount of

fiber content which make it functional product good for diabetic people.

Also important flavonoids present in grains taken for formulating breads, like rutin, quercetin, epicatechin and chlorogenic acid are quantified from HPLC. These four quantified compounds have antidiabetic, antioxidant properties which will impart health benefit to consumers in the form of breakfast meal i.e. bread. In HPLC analysis it was observed that not all flavonoids decrease after baking. Analysis of functional properties of flours like swelling index, of both the composite flour (RWI & RWII) was less than the control flour, which indicates that the protein and fiber content of composite flour was more. Also the foaming capacity of flour indicated that the globular proteins present in flours are highly ordered. The baking loss was found to be in the acceptable range.

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