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Supriya Rattan
Food Processing Laboratory, University School of Biotechnology, GGS Indraprastha University, Dwarka-110078, India

Gouri Satpathy
Food Processing Laboratory, University School of Biotechnology, GGS Indraprastha University, Dwarka-110078, India

Rajinder K. Gupta
Food Processing Laboratory, University School of Biotechnology, GGS Indraprastha University, Dwarka-110078, India

Correspondence:
Rajinder K. Gupta
Food Processing Laboratory, University School of Biotechnology, GGS Indraprastha University, Dwarka-110078, India
Email: rkg67ap@yahoo.com
Tel: +91-11-25302321,

Formulation of biscuits with *Glycyrrhiza glabra* root extract, *Ocimum sanctum* and *Pimpinella anisum* extract added as a nutraceutical

Supriya Rattan, Gouri Satpathy, Rajinder K. Gupta

Abstract
A formulation of biscuits with *Glycyrrhiza glabra* (liquorice) root extract, *Ocimum sanctum* (holy basil) and *Pimpinella anisum* (anise) extract was prepared for enhancing the healthy beneficial usage of biscuits as nutraceutical, in conditions like cough or sore throat with a simultaneous attainment of the other health benefits. Both extracts and biscuits were tested for total flavonoids, total phenolics, alkaloids, and antioxidants and the liquorice extract was found to be rich in antioxidants followed by Basil and Anise extract. Liquorice extract was also found to be rich in total phenolics (3450 µg per 100 mg extract) while basil extract showed maximum amount of flavonoids *i.e.*, 16,880 µg per 100 mg extract. The biscuits were also tested for various nutritional and sensory components and were found to be a good source of protein (8.4%) & dietary fibers (4.8%) as compared to the normal wheat biscuits.

Keywords: Biscuits, Basil, Liquorice, Anise, GC-MS, Phytochemical, Antioxidant, Nutritional, Dietary fiber, Protein, Extracts.

1. Introduction
According to a survey conducted by WHO, 1993, about 80% of patients in India, 85% in Burma and 90% in Bangladesh obtain their medical treatment through traditional system of medicines [1]. Liquorice root contains triterpenoid saponins (4–20%), a mixture of potassium and calcium salts of glycyrrhizic acid (also known as glycyrrhizinic acid) which is 50 times as sweet as sugar [2]. Several studies in past years have already depicted the health benefits of *Glycyrrhiza glabra*, *Ocimum sanctum* and *Pimpinella anisum* and researchers have also proved the antioxidant properties of ethanol extract of holy basil leaves [3]. Basil is associated with numerous health benefits as it contains high amounts of (E)-beta-caryophyllene (BCP), which may found its use in treating arthritis and inflammatory bowel diseases, and is also incredibly rich in vitamin A, vitamin K, vitamin C, magnesium, iron, potassium, and calcium. Other *in vivo* and clinical studies have reported beneficial effects of both Liquorice and glycyrrhizin consumption including anti-ulcer, anti-viral, and hepatoprotective responses. According to Hicham *et al.* [4], *O. basilicum* may contain polar products that can lower down the plasma lipid concentrations and thus can prevent hyperlipidemia and related cardiovascular diseases. Extracts of *O. sanctum* leaves inhibit the proliferation, migration, invasion, and induced apoptosis of Pancreatic cancer cells *in vitro* [5]. In a study, İlhami [6] has reported the antioxidant and antimicrobial activities of water and ethanol extracts of anise (*Pimpinella anisum* L.) seed. The aqueous extract of *G. glabra* was found to inhibit *in vivo* and *in vitro* proliferation of Ehrlich ascites tumor cells, and angiogenesis in *in vivo* peritoneal and chorioallantoic membrane assays [7].
In this study, the biscuits were formulated with the extracts of *G. glabra*, *O. sanctum* and *P. anisum* in different optimized concentrations. These formulated biscuits were tested for phytochemical, antioxidant, and nutritional contents in order to determine their feasibility as a potential nutraceutical containing both taste and health benefits.

2. Materials and Methods
2.1 Materials
Anise and basil extracts were purchased from Navchetna Kendra (New Delhi, India) while liquorice extract (20% by HPLC) was procured from Surya Herbal Extracts (Hyderabad, India).

Several other essential baking components including wheat flour, barley flour, edible oil etc were obtained from local market. Ethanol, Methanol, Gallic Acid, Petroleum Ether, Folin-Ciocalteau Reagent, Sodium Carbonate, Dimethyl Sulfoxide (DMSO), Sodium Nitrite, Catechin, etc. were procured from Fluka (Fluka, Switzerland) and Sigma- Aldrich (Sigma- Aldrich, St. Louis, MO, USA). HPLC grade water and solvents were purchased from Rankem (RFCL ltd, India).

3. Method

3.1 Formulation of biscuits with liquorice, basil and anise extracts

The biscuits were prepared using different formulation ratios of root extract of *Glycyrrhiza glabra* (liquorice), *Ocimum sanctum* (holy basil) and *Pimpinella anisum* (anise) extract as shown in Table 1 and were baked in a local bakery using a standard concentration of sugar and edible oil. A constant wheat flour concentration was kept in all the biscuit preparations except the *BF1* variant that had the mixed flour containing wheat and barley flour in 4:1 ratio, respectively.

Table 1: Different concentrations of extracts used for formulation of biscuits.

Biscuit Code No.	Flour used	Amount of dough mixture(g)	Amount of Anise extract(g)	Amount of Basil extract(g)	Amount of Liquorice extract(g)	Total amount of extracts(g)
WF 1	Wheat flour	200	0.5	0.35	1.5	2.35
WF 2	Wheat flour	200	0.5	0.5	0.5	1.5
WF 3	Wheat flour	200	1.0	1.0	1.0	3.0
BF 1	Wheat flour and Barley flour (4:1)	200	0.5	0.35	0.75	1.60

3.2 Determination of total phenolics, total flavonoids, and total alkaloids in raw extracts and formulated biscuits

Folin-Ciocalteu reagent was used to determine total phenolics content [8-9] in ethanolic-water extracts of raw extracts of liquorice, basil and anise and formulated biscuits (*WF1* and *BF1)*. It was expressed in terms of one milligram gallic acid equivalents (GAE) per gram of dry extract, and the absorbance was measured at 765 nm, using a UV–visible spectrophotometer (Shimadzu UV-1601PC, Japan). Results were measured on a dry weight basis as µg gallic acid equivalents/mg of sample. Each determination was performed in triplicate, and was repeated thrice [10].

Total flavonoids content was also determined for ethanolic extracts of all the samples using aluminium chloride colorimetric method [11-12], and expressed in terms of mg catechin equivalents (CE)/g of dry extract. Total alkaloids content was determined as per the method illustrated by Harborne [13].

3.3 Determination of reducing power of raw extracts and formulated biscuits by FRAP assay

Total antioxidant activity was measured by ferric reducing antioxidant power (FRAP) assay as depicted by Benzie and Strain [14] in which a standard solution was prepared by using several range of dilutions of butylated hydroxytoluene (BHT) from 10 µL to 100 µL. All the samples of ethanolic extracts of liquorice, basil, anise, and formulated biscuits (*WF1* and *BF1)* were then analysed by mixing FRAP solution (900 µL) with 100 µL of extract. Thereafter samples were incubated at 37 °C for 4 min and their absorbance was measured at 593 nm.

3.4 ABTS assay of raw extracts and formulated biscuits

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) Assay was performed for the determination of anti-oxidative activity from the decrease of absorbance at 734 nm with ascorbic acid as a calibrator and was expressed as ascorbic acid equivalents. Thus, ABTS reagent was prepared by dissolving 16 mg Potassium persulfate and 96 mg ABTS in 25 mL of 0.01 M Phosphate buffer saline (PBS), and then keeping it for 16 hours in dark at room temperature. Thereafter, the absorbance of ABTS reagent was set at 0.7 ±0.1 by diluting it with PBS at 734 nm, and 980 µl of this ABTS reagent was then added to 20 µL sample. After 3min incubation at room temperature, the final absorbance was taken at 734 nm against the ascorbic acid as a standard.

3.5 GC-MS analysis of ethanolic extracts of liquorice, basil and anise for secondary metabolites

Further analysis of prepared ethanol-water extracts was conducted by GC-MS during which 1µL methanolic extract injections were performed by split injection (1:20) at 280 ^{0}C. The oven was programmed at several temperature including 65 $^\circ$C (for 5 min), 15 ^{0}C to 180 ^{0}C (for 10 min), 5 ^{0}C to 280 ^{0}C (for 15 min). Helium was used as carrier gas. An Agilent 5975B mass spectrometric detector (MSD) was used in the scan mode (m/z 35-1050) for all samples. Screening of volatiles and semi volatiles was performed using the automatic RTL screener software in combination with the Agilent NIST'05 library. Gas saver option was turned off, transfer line temperature was set to 300 ^{0}C, solvent delay was 3 min, ion source and quadrupole temperature were 230 ^{0}C and 150 ^{0}C, respectively. Those compounds were considered having ≥75% matching with NIST library.

3.6 Sensory analysis of formulated biscuits

Samples of freshly prepared biscuits, and baked biscuits with 30 days of storage period were subjected to a sensory test. Characteristic flavour, aroma, and texture were identified using a 9 points hedonic scale (1 = disliked very much; 9 = liked very much). Samples were labelled with code numbers and presented in a random sequence to the members of panel. The panelists were instructed to drink water after every sample, and were not allowed to comment during evaluation for preventing peer-influence on other panelists. Post-evaluation, they were also asked to write down their comments in a questionnaire provided to them.

3.7 Color value of formulated biscuits

The color value of biscuits was determined using Hunter Lab Color spectrometer, based on three elements: hue, chroma and value (lightness). By describing a colour using these three attributes, we can accurately identify and differentiate a particular colour from other. Thus, the values of L, a and b were calculated by calibrating in the black and white range and hue angle and chroma were calculated for the biscuits where, "L" defines lightness, "a" denotes the red/green value and "b" the yellow/blue value.

3.8 Determination of nutritional constituents of formulated biscuits

Moisture content was determined by drying the samples in oven at 105 °C for 5 h [15]. Similarly, ash analysis was carried out by burning the sample in muffle furnace at 500 °C for 5 h [16]. Protein content was evaluated by multiplying the nitrogen content by the coefficient 6.25 using kjeldahl method [17] whereas for fat content determination, petroleum ether was used as solvent with continuous stirring at 25 °C for 2 days [17]. The amount of total carbohydrates was calculated with the following formula:

Total carbohydrates (% fresh weight)
= 100 - moisture (%) - protein content (% fresh weight) - crude fat (% fresh weight) - ash (% fresh weight) and reported as total carbohydrates in g per 100 g dry mass. The caloric value per 100 g of dry mass was calculated according to the system of Atwater, namely:

kcal = (3.36 × % protein fresh weight) + (3.60 × % total carbohydrate fresh weight) + (8.37 × % fat fresh weight).

4. Results and Discussion

4.1 GC-MS analysis of extracts

The GC-MS analysis of methanolic extracts of anise, basil and liquorice are shown in table 2, 3, and 4 respectively. n Hexadecanoic acid has an anti-inflammatory property [18]. Isosorbide helps mainly to prevent angina chest pain [19]. Phytol can activate some enzymes within the body that have strong positive effects on insulin level [20]. Results of GC-MS profile of liquorice extract also exhibited some essential components like benzofuran, isosorbide, D-allose, hexadecanoic acid etc. Methylparaben, an anti-fungal, anti-irritant agent and water-soluble anti-microbial was also among such compounds [21]. D-Allose helps in the protection of neurons, thus, can reduce the occurrence of various neurodegenerative diseases such as Parkinson's disease and prion diseases [22-23].

Table 2: Compounds identified by GC-MS analysis of anise methanolic extract.

S. No.	Compound Name	CAS No.	RT(min.)	Area (%)
1.	n-Hexadecanoic acid	000057-10-3	20.392	7.96
2.	9,12-Octadecadienoic acid (Z,Z)-cyclododecyne	000060-33-3	22.310	32.20
3.	Octadecanoic acid	000057-11-4	22.422	6.96
4.	9,12-Octadecadienoic acid	000060-33-3	22.546	2.86
5.	Cyclooctene-3-ethenyl	002213-60-7	22.703	1.12
6.	1-Hexadecyne	000629-74-3	22.826	1.64
7.	9-Octadecenal	002423-10-1	24.878	4.81
8.	Nonanoic acid	055268-58-1	26.706	3.81

Table 3: Compounds identified by GC-MS analysis of basil methanolic extract

S. No.	Compound Name	CAS No.	RT(min)	Area (%)
1.	Ethyl Acetate	000141-78-6	3.479	2.21
2.	Dimethyl Sulfoxide	000067-68-5	4.635	3.11
3.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	028564-83-2	9.839	1.33
4.	Isosorbide	000652-67-5	12.239	2.25
5.	Naphthalene, 1,2,3,4,4a,5,6,8a-oct ahydro-4a,8-dimethyl-2-(1-methylet henyl)-,[2R(2.alpha.,4a.alpha.,8a.beta.)	000473-13-2	17.252	1.85
6.	6-Isopropenyl-4,8a-dimethyl-1,2,3 5,6,7,8,8a-octahydro-naphthalen-2-ol	1000189-10-2	17.992	1.43
7.	Hexadecanoic acid, methyl ester	000112-39-0	19.955	7.50
8.	9,12-Octadecadienoic acid (Z,Z)-,methyl ester	000112-63-0	21.603	7.82
9.	Phytol	000150-86-7	21.760	1.22
10.	Octadecanoic acid, methyl ester	000112-61-8	21.861	1.73
11.	9,17-Octadecadienal	056554-35-9	22.041	15.17
12.	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	001191-41-9	22.265	1.49
13.	Z,E-7,11-Hexadecadien-1-yl acetate	051607-94-4	26.527	6.11

Table 4: Compounds identified by GC-MS analysis of liquorice methanolic extract

S. No.	Compound Name	CAS No	RT(min)	Area (%)
1.	3-Pyridinol	000109-00-2	9.188	1.60
2.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	028564-83-2	9.816	2.07
3.	Benzofuran	000496-16-2	11.106	3.82
4.	Isosorbide	000652-67-5	12.227	6.26
5.	2-Methoxy-4-vinylphenol	007786-61-0	12.665	6.81
6.	Methylparaben	000099-76-3	14.616	2.46
7.	D-Allose	002595-97-3	15.110	10.98
8.	Octanal,2-(phenylmethylene)-	000101-86-0	18.194	1.02
9.	Hexadecanoic acid, methyl ester	000112-39-0	19.943	4.67
10.	n-Hexadecanoic acid	000057-10-3	20.302	8.25
11.	Dibutyl phthalate	000084-74-2	20.381	1.92

4.2 Sensory analysis of formulated biscuits

The most acceptable biscuit formulation contained 0.75% Liquorice, 0.175% basil and 0.3% anise extracts in 100 g wheat flour dough mixture, and 0.37% liquorice, 0.25% anise and 0.25% basil extracts in 100g barley flour and wheat flour combination (1:4). The shelf-life study of biscuits revealed that for up to 60 days of storage, the overall acceptability of biscuits was in the category of "like moderately."

Hence, these two formulations (WF1 and BF1) were tested for total concentration of phenolics and flavonoids, antioxidant activity, and nutritional analysis.

4.3 Total phenolic content of extracts and formulated biscuits

The total phenolic content (expressed in terms of GAE and yield (%) of the extracts) was found to be maximum for liquorice extract. The total phenolic content was calculated using the linear equation based on the calibration curve of gallic acid (see Table 5).

Table 5: Total phenolics estimation of anise, basil, and liquorice extracts, and formulated biscuits (WF1 and BF1).

S. No.	Extract	Concentration of Total Phenolics (GAE per 100 mg)
1.	Anise	2765.663 µg
2.	Basil	2322.29 µg
3.	Liquorice	3450.0 µg
4.	WF1	575.9 µg
5.	BF1	385.0 µg

4.4 Total flavonoid content of extracts and biscuits

Total flavonoids were measured in terms of Catechin equivalent (CE) using the linear equation based on the calibration curve of catechin, and was found to be rich in basil extracts as compared to anise and liquorice. The total flavonoids were expressed as micrograms of catechin equivalents (CE) per mg of dried fraction and are shown in table 6.

Table 6: Total flavonoids estimation of anise, basil, and liquorice extracts, and formulated biscuits (WF1 and BF1).

S. No	Extracts	Concentration of Total Flavonoids (CE per 100 mg)
1.	Anise	380.44 µg
2.	Basil	16,880 µg
3.	Liquorice	297.33 µg
4.	WF1	169.6 µg
5.	BF1	168.71 µg

4.5 Determination of alkaloids

Alkaloids have diverse and important physiological effects on humans and other animals. Well-known alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine. Concentrated ammonium hydroxide was added to the raw extracts and biscuits samples for detecting the presence of alkaloid in it but the absence of precipitates in newly formed mixture had signified that none of the samples contained alkaloids.

4.6 Determination of reducing power by FRAP assay

In FRAP assay, reduction of the ferric-tripyridyltriazine to the ferrous complex forms an intense blue colour which can be measured at a wavelength of 593 nm. The intensity of the colour is related to the amount of antioxidant reductants in the samples. Since, FRAP is based on the principle of ferric to ferrous ion reduction at low pH that causes a colored ferrous-tripyridyltriazine complex to form, therefore the FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration (see table 7).

Table 7: Total antioxidant estimation of anise, basil, and liquorice extracts using FRAP assay.

S. No.	Extracts	Concentration of Antioxidants (BHT equivalent per 100 mg)
1.	Anise	8.046 µg
2.	Basil	10.7548 µg
3.	Liquorice	11.9544 µg
4.	WF1	6.28 µg
5.	BF1	7.305 µg

4.7 Determination of antioxidant activity by ABTS assay

Antioxidant capacity of the extracts was evaluated against

ascorbic acid as percent inhibition of ABTS free radicals. ABTS radical is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Thus, antioxidant activity of anise, basil, and liquorice extracts was calculated by plotting a line plot between the concentration in mg/ml and % scavenging (Fig.1). Since, the test was done in triplicate, so % scavenging of these three readings was calculated, followed by the calculation of an average value of % scavenging. Then, IC_{50} value was calculated by locating the concentration at which 50% scavenging was taking place. The results showed that IC_{50} value was minimum for liquorice extract indicating that this extract was rich in antioxidants, and very less quantity of liquorice is required for scavenging of oxidants. Similarly, basil extract had shown IC_{50} value at a concentration of 10 mg/ml, and IC_{50} value for anise was found at the concentration of 30 mg/ml (Table 8).

Table 8: Showing IC_{50} value obtained by ABTS assay of anise, basil, and liquorice extracts in mg/ml of extract.

S. No.	Extracts	IC_{50} Value
1.	Anise	30 mg/ml
2.	Basil	10 mg/ml
3.	Liquorice	4 mg/ml

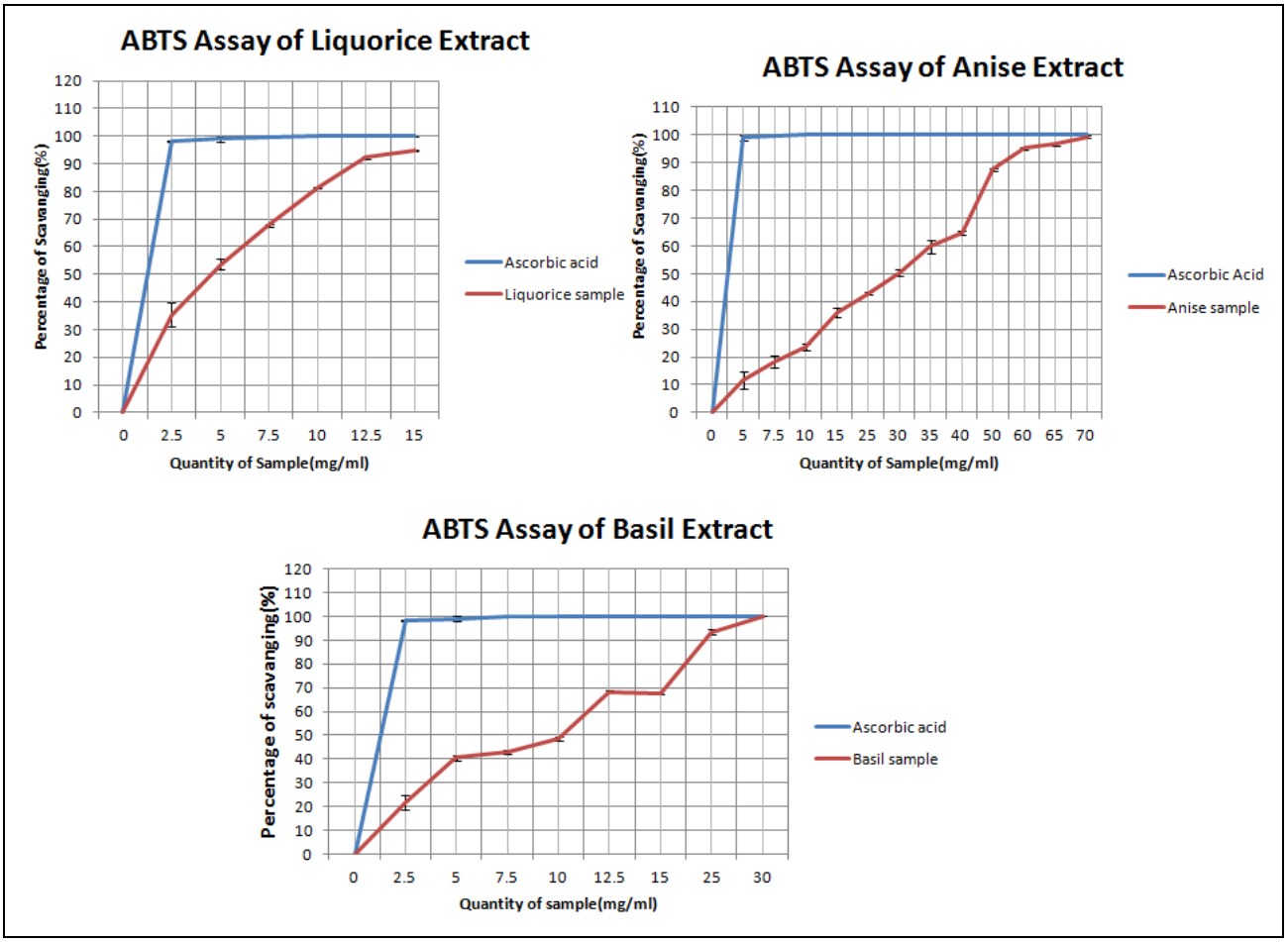

Fig 1: Graphs showing % scavenging shown by ABTS assay of anise, basil and liquorice.

4.8 Determination of Color value of Biscuits

The color value was determined in terms of hue angle and chrome so as to differentiate the color from all others and assign it a numeric value. Hue is how we perceive an object's color and chrome describes the vividness or dullness of a color, in other words, how close the color is to either gray or the pure hue. Table 9 shows the results of color value determination.

Table 9: Values of "L", "a", "b", hue angle and chroma for different biscuit samples.

Biscuit Code No.	Value of "L"	Value of "a"	Value of "b"	Hue angle	Chrome
WF 1	60.145	7.39	22.765	72.01º	23.93
WF 2	56.05	6.51	20.09	72.04º	21.11
BF 1	56.91	7.86	21.793	70.16º	23.16

4.9 Determination of nutritional content of formulated biscuits

The principal characteristic which differentiates biscuit from other bakery products is the presence of low moisture content (5% max). Although water is added in dough-making process but unbound water evaporates rather easily during the baking process at high temperatures (as high as 230 °C), and water bound to some functional groups of the molecules of substances present in the bakery products may also get partly released at such high temperatures, and thus helps in colouring the surface of the final products [24]. The moisture content estimated for formulated biscuits with wheat flour (WF 1) and a combination of wheat and barley flour (BF 1), after drying the 5 g samples in oven at 105 °C for 5 h was found to be 1.16 % for WF1 and 1.31% for BF1 formulation. The total fats were found to be more in biscuits with barley content as compared to that with wheat flour. The dietary fiber content in BF1 formulated biscuits were found to be 4.8% which was very high as compared to the normal wheat biscuits containing 1.5-2% dietary fiber [25] content. Total protein content in BF1 and WF1 formulated biscuits was observed as 8.4% and 6.65%, respectively. WF1 biscuits have shown to possess comparatively more amount of carbohydrate content than BF1 variant. Higher amount of caloric value was also determined in both the blends of biscuits as compared to the normal wheat biscuits [25].

Table 10: Showing nutritional content analysis done for formulated biscuits WF1 and BF1.

Nutritional Constituent (per 100 g)	WF 1 (Biscuit containing wheat flour)	BF 1 (Biscuit with barley and wheat flour in 1:4 ratio
Moisture	1.168	1.318
Ash	0.052	0.38
Total fat	22.182	28.064
Total protein	6.65	8.4
Total carbohydrates	69.94	61.838
Caloric value	458.92 kcal	484.54 kcal

5. Conclusion

In the end, we can conclude that the extracts used for formulating the biscuits are rich in antioxidants and flavonoids, and act as a flavour enhancer along with a medicinal benefit. These nutraceutical biscuits containing barley-wheat formulations are also rich in dietary fiber content (8.4%) as compared to the normal wheat biscuits which have 1.5-2% dietary content. This product has come up with the goodness of basil in providing DNA protection and anti-bacterial properties, liquorice for the treatment of coughs, colds, and other bronchial irritations, and anise as a flavour enhancer because of the high concentration of anethole. The major active constituents called flavonoids found in basil provide protection while the enzyme-inhibiting effect of the eugenol, present in it, provides an anti-inflammatory property that enables its potential application in treatment of rheumatoid arthritis or inflammatory bowel conditions. Powdered liquorice is also well thought-out in ayurvedic medicine as an excellent remedy for hyperacidity and clinical tests has proven its significance in relieving pain, discomfort and other symptoms caused by acid reflux in the stomach. Thus, this amalgamation of a number of health benefits in the form of a nutraceutical biscuit can tickle the taste buds of the consumers and influence them for consuming it for a healthy well-being.

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