

**PHYTOCHEMICAL CHARACTERIZATION OF BLACK SOYBEAN (*GLYCINE
MAX (L.) MERRILL*) AND ITS VALUE ADDITION**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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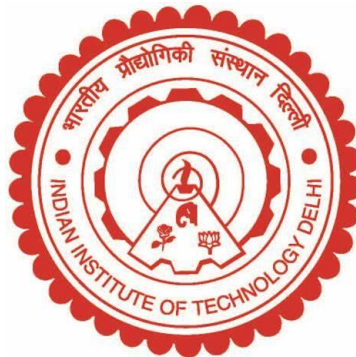
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CERTIFICATE

This is to certify that the thesis entitled “**Phytochemical characterization of black soybean (*Glycine max (L.) Merrill*) and its value addition**” being submitted by **Mr. Ankur Kumar** to the **Indian Institute of Technology Delhi** for the award of “**Doctor of Philosophy**” is a record of bonafide research work carried out by him. He has worked under our guidance and supervision and has fulfilled the requirements for the submission of this thesis. To the best of our knowledge, the results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree or diploma.

(Prof S N Naik)

Centre for Rural Development &Technology
Indian Institute of Technology Delhi

(Prof Jatindra K Sahu)

Centre for Rural Development &Technology
Indian Institute of Technology Delhi

Date:

Place: New Delhi

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Ankur Kumar

Date:

Place: New Delhi

ABSTRACT

Black soybean (*Glycine max* (L.) Merrill) locally known as *bhat*, *kalabhat* or *bhatmaas* is an underutilized leguminous crop confined to the Indo-Himalayan regions. Black soybean has unique properties owing to its black hull, making it different from yellow and green soybean. There is wide variability in the *black soybean* in terms of seed weight, length and width and other quality parameters. There is a need to explore these legumes to assess their genotype diversity for further utilization in food and seed improvement programs. With this background, the present research was designed to validate the nutritional claim in the Indo-Himalayan *black soybean* population, its lipid quality assessment, effect of cooking methods on antinutritional factors, and development of value-added products including its nutritional quality and sensory evaluation.

Black soybean specimen samples from five geographical locations of Indo-Himalayan regions (BSALS & BSALB, BSNDD, BSRMN and BSGRP - samples collected from Almora, Dehradun, Ramnagar and Nainital regions, respectively) were collected. The samples were authenticated and analyzed in terms of seed dimensions and proximate, mineral and amino acid compositions to evaluate the seed variability. Polyphenolic compounds extracted using a central composite design by response surface methodology (RSM) using ultra-sound assisted (UAE) extraction techniques with three process variables (solid to solvent ratio, ultrasound amplitude and time) and three responses i.e. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, total phenolic content (TPC) and total flavonoid content (TFC). The UAE optimized samples were analyzed to target three β -glucoside, three aglycon isoflavones, one anthocyanin cyanidin-3-glucoside and three polyphenols (gallic, caffeic and quercetin) using HPLC technique. The lipid quality was assessed among the samples for oil yield, β -carotene, tocopherols, and unsaponified matter. Fatty acid and flavor estimation was performed using GC and GC-MS-HS. Supercritical fluid and Soxhlet extractions were compared for different oil quality characteristics of *black soybean*. Effects of microwave cooking (400, 800 and 1200 W for 10 min), pressure vessel (48.26 kPa, 68.95 kPa, and 82.73 kPa, respectively), and conventional clay pot cooking at 70°C, 90°C and 110°C on raffinose and phytic acid - two antinutrients in *black soybean* were analyzed. Further, black soybean flour was utilized for the preparation of cookies.

There was a significant variation ($p < 0.05$) in the seed weight (0.084 to 0.158 g) among the seed samples. The BSALS seeds had the lowest width and weight comparatively. The protein content in all samples were measured to be in the range 31.1 to 39.7 g/100g with the maximum in small oval shape BSALS seeds, a nonsignificant difference ($p > 0.05$) among the black soybean samples revealed the variance in the proximate compositions irrespective of the seed dimensions. Sucrose was observed in the range of 3.84 to 5.02 g/100g with a minimum concentration in BSALS and maximum in BSGRP seeds. FTIR characterization reported three major peaks at 2835, 1640, and 1015 cm^{-1} wavenumber and reported the presence of aldehyde and ketone compounds. Protein quality assessment for individual amino acids (AAs) reported sixteen AAs, of which seven were classified as essential and nine as non-essential AAs. On differentiating the AA values with the physical characteristics of *black soybean* varieties, it was found that BSGRP samples possessed the minimum AA and the maximum was noted in BSALS samples. Among essential AAs, lysine was comparatively higher in BSALS seed samples (2.32 g/100g).

Sulphur containing amino acid methionine and cysteine had low levels in all samples. Co and Cr were not detected in any of the samples among eleven mineral elements. Micronutrients were comparatively higher in BSALS seed samples.

The UAE extraction conditions (ultrasound power, time and solid-to-solvent ratio) for polyphenols (DPPH assay, TPC and TFC) were optimized using a central composite design by response surface methodology. A quadratic model with F-values of 29.38 and 8.77 was suggested for DPPH assay and TPC, respectively. The extract optimized using UAE process were analyzed using HPLC-UV for estimation of isoflavones and anthocyanin content. Total isoflavones significantly ($p < 0.05$) varied from 85.41 to 199.44 mg/100g with the highest concentration in BSALS samples. β -glucoside isoflavones were reported 85.73 to 93.18% of total isoflavones. Cyanidin-3-glucoside (C3G) varied from 29.23 to 71.85 mg/100 g with the maximum in BSGRP seed samples and lowest in BSALS samples. The BSALS samples had a lower oil content (about 20.53%), and maximum β -carotene content (1305 μ g/kg), while the oil yield was about 25.64% in BSALB seed samples. γ -tocopherol was highest, followed by δ -tocopherol and α -tocopherol among all seed samples. The oil was consisted of five major fatty acids: methyl palmitate (9.79 to 10.87%), methyl stearate (3.12 to 4.26%), cis 9-oleic acid methyl ester (19.46 to 25.4%), methyl linoleate (54.63 to 57.44%) and, methyl linolenate (5.81 to 9.75%) with the non-significance distribution. Lipid fractions reported 46 volatile flavor compounds (VFCs) as identified in GC-MS/HS analysis. There was a wide difference in the composition of these compounds among individual lipid fractions. Supercritical fluid extraction (SFE) reported oil yield of 19.1%, while the Soxhlet produced 23.67% oil yield. Unsaponified matter and carotenoid content in SFE technique were slightly higher (2.73% and 1.259 mg/kg) than the Soxhlet extraction (2.57% and 1.139 mg/kg), indicating that SFE oil exhibited comparatively higher antioxidant activities. Flavor 1,2-Propanediol, 3-benzyloxy-1,2-diacetyl- was observed higher in SFE.

The conventional clay pot and pressure vessel cooking methods reported to reduce about 32.50% and 75.0% of phytic and raffinose, respectively, while cyanidin-3-glucoside was significantly degraded in all the treatments. *Black soybean* flour-based gluten-free cookies were prepared to realize the consumers perspective for the black soybean derived food product. The average sensory score for flavor and color were 7.8 and 8.2. The texture, taste, and mouthfeel scores were 8.1, 8.0 and 8.1, respectively on a 9-point hedonic scale and showed good consumer acceptability.

In conclusion, the present study reports the scientific validation of local *black soybean* for phytochemical and lipid composition. This profiling would help developing various value added and protein rich nutritious food products exploring utilization of underutilized *black soybean* in local and global markets.

Keywords: Antinutrients; *Black soybean*; Lipid characteristics; Antinutritional factors; Sensory qualities; Ultrasound assisted extraction

सारांश

काला सोयाबीन (*ग्लाइसीन मैक्स (एल.) मेरिल*), जिसे स्थानीय भाषा में भट, कालाभट, भातमास के रूप में जाना जाता है भारत-हिमालयी क्षेत्रों तक सीमित कम उपयोग वाली फलीदार फसलों में से एक है। इसके काले छिलके के कारण इसमें अद्वितीय गुण हैं, जो कि पीले और हरे सोयाबीन से अलग होते हैं। काले सोयाबीन में बीज वजन, लंबाई और चौड़ाई और अन्य गुणवत्ता मापदंडों के मामले में काफी विविधताएं हैं। खाद्य और बीज सुधार कार्यक्रमों में उपयोग के लिए इनकी जीनोटाइप विविधता का आकलन करने के लिए इन फलियों के मूल्यांकन करने की आवश्यकता है। इस पृष्ठभूमि के साथ, वर्तमान शोध को भारत-हिमालयी काली सोयाबीन में पोषण एवं अन्य फाइटो केमिकल संबंधी दावे को मान्य करने; लिपिड गुणवत्ता मूल्यांकन; पोषण विरोधी कारकों पर विभिन्न खाना पकाने के तरीकों का प्रभाव, और मूल्यवर्धित उत्पाद विकसित कर इसकी गुणवत्ता और संवेदी मूल्यांकन करने के उद्देश्य से प्लान किया गया है।

भारत के हिमालयी क्षेत्रों से काले सोयाबीन के विभिन्न प्रकार के पांच नमूने एकत्र किए गए जो की क्रमश बीएसएएलएस (अल्मोड़ा), बीएसएएलबी (अल्मोड़ा), बीएसएनडीडी (देहरादून), बीएसआरएमएन (रामनगर), और बीएसजीआरपी (नैनीताल) थे। नमूनों का प्रमाणीकरण एवं विश्लेषण कर बीजों का मूल्यांकन बीज आयाम, समीपस्थ, सुक्रोज, खनिज और अमीनो एसिड संरचना के संदर्भ में किया गया। पॉलीफेनोलिक यौगिकों के निष्कर्षण को तीन प्रक्रिया चर (ठोस से विलायक अनुपात, अल्ट्रासाउंड आयाम और समय) और तीन प्रतिक्रियाओं यानी २,२-डिफेनिल-१-पिक्रीलहाइड्रज़िन (डीपीपीएच) परख, कुल फेनोलिक सामग्री (टीपीसी) और कुल फेनोलिक सामग्री (टीएफसी) अल्ट्रा-साउंड असिस्टेड (यूई) निष्कर्षण प्रतिक्रिया सतह पद्धति (आरएसएम) द्वारा केंद्रीय समग्र डिजाइन का उपयोग करके अनुकूलित किया गया। निष्कर्षण अनुकूलन के बाद, तीन β -ग्लूकोसाइड, तीन एग्लीकॉन आइसोफलेवोन्स, एक एंथोसायनिन साइनाइडिन-३-ग्लूकोसाइड और तीन पॉलीफेनोल्स (गैलिक, कैफिक और क्वेरसेटिन) को लक्षित करने के लिए एचपीएलसी द्वारा नमूनों का विश्लेषण किया गया। तेल उपज, β -कैरोटीन, टोकोफेरोल्स और असैपोनिफाइड पदार्थ के लिए सभी नमूनों में लिपिड गुणवत्ता मूल्यांकन किया गया। जीसी और जीसीएमएस-एचएस का उपयोग करके फैटी एसिड और स्वाद अवयवों का अनुमान लगाया गया। काले सोयाबीन की विभिन्न तेल गुणवत्ता विशेषताओं के लिए सुपरक्रिटिकल और सॉक्सलेट निष्कर्षण की तुलना की गई। खाना पकाने के प्रभाव का अध्ययन विभिन्न खाना पकाने की प्रक्रियाओं का उपयोग करके किया गया। १० मिनट के लिए ४००, ८०० और १२०० वाट पर माइक्रोवेव खाना पकाने, दबाव और पारंपरिक मिट्टी के बर्तन में कम मध्यम और उच्च तापमान एवं दबाव द्वारा रैफिनोज और फाइटिक एसिड पर होने वाले प्रभाव को देखा गया, जिन्हें काले सोयाबीन में एंटीन्यूट्रिएंट्स माना जाता है। इसके अलावा, काले सोयाबीन के आटे का उपयोग कुकी बनाने में किया गया।

बीज वजन (८४.० से १५८.० मिलीग्राम) में महत्वपूर्ण भिन्नता ($p < 0.05$) थी। बीएसएएलएस बीज की तुलनात्मक रूप से सबसे कम चौड़ाई और वजन होता है। प्रोटीन की मात्रा ३१.१ से ३९.७ ग्राम / १०० ग्राम पायी गयी। प्रोटीन की अधिकतम मात्रा छोटे अंडाकार आकार के बीएसएएलएस बीज में थी। काले सोयाबीन के नमूनों के बीच निरर्थक अंतर ($p > 0.05$) ने दिखाया की समीपस्थ रचनाओं में भिन्नता, बीज के आयामों के निरपेक्ष है। सुक्रोज ३.८४ से ५.०२ ग्राम/१०० ग्राम की सीमा में पाया गया। छोटे बीएसएएलएस बीज में यह न्यूनतम और मध्यम आकार के बीएसजीआरपी बीज में अधिकतम पाया गया। एफटीआईआर लक्षण वर्णन से २८३५, १६४०, और १०१५ cm^{-1} तरंग संख्या पर तीन प्रमुख चोटियों की जानकारी मिली, जिससे एल्डिहाइड और कीटोन यौगिक की उपस्थिति का पता चला। वैयक्तिक अमीनो एसिड (एए) के लिए प्रोटीन गुणवत्ता मूल्यांकन ने सोलह एए की सूचना दी, जिनमें से सात को आवश्यक एए और नौ को गैर-आवश्यक एए के रूप में वर्गीकृत किया गया। काले सोयाबीन की किस्मों की भौतिक विशेषताओं के साथ एए मूल्यों को अंतर करने पर यह पाया गया कि बीएसजीआरपी (गोलाकार आकार के मध्यम आकार के) बीज में न्यूनतम एए थे और अधिकतम बीएसएएलएस (छोटे अंडाकार आकार के बीज) में नोट किये गए। आवश्यक एए में, बीएसएएलएस (अंडाकार आकार, सबसे कम भार वाले) बीज में लाइसिन (२.३२ ग्राम/१०० ग्राम) तुलनात्मक रूप से अधिक था। सल्फर युक्त अमीनो एसिड मेथियोनीन और सिस्टीन के सभी नमूनों में निम्न स्तर रहा। ग्यारह खनिज तत्वों में से किसी भी नमूने में कोबाल्ट और क्रोमियम नहीं पाए गए। छोटे अंडाकार आकार के बीएसएएलएस बीज के नमूनों में सूक्ष्म पोषक तत्व तुलनात्मक रूप से अधिक थे।

पॉलीफेनोल्स (डीपीपीएच परख, टीपीसी और टीएफसी) के लिए यूई निष्कर्षण की स्थितियों (अल्ट्रासाउंड शक्ति, समय और ठोस से विलायक अनुपात) को प्रतिक्रिया सह पद्धति द्वारा केंद्रीय समग्र डिजाइन का उपयोग करके अनुकूलित किया गया था। क्रमशः डीपीपीएच और टीपीसी के लिए २९.३८ और ८.७७ के एफ मूल्यों के साथ द्विघात मॉडल का सुझाव दिया गया था। आइसोफ्लेवोन्स और एंथोसायनिन सामग्री के आकलन के लिए एचपीएलसी-यूवी का उपयोग करके यूई प्रक्रिया द्वारा अनुकूलित अर्क का विश्लेषण किया गया। छोटे अंडाकार आकार के बीएसएएलएस नमूनों में उच्चतम सांद्रता के साथ कुल आइसोफ्लेवोन्स महत्वपूर्ण रूप से ($p < 0.05$) ८५.४१ से १९९.४४ मिलीग्राम / १०० ग्राम तक भिन्न होते हैं। β -ग्लूकोसाइड आइसोफ्लेवोन्स कुल आइसोफ्लेवोन्स के ८५.७३ से ९३.१८% तक दर्ज किये गए। साइनाइडिन-३-ग्लूकोसाइड (सी३जी) २९.२३ से ७१.८५ मिलीग्राम/१०० ग्राम के बीच भिन्न था जो की बीएसजीआरपी बीज नमूनों में अधिकतम और बीएसएएलएस नमूनों में सबसे कम था। बीएसएएलएस के नमूनों में कम तेल सामग्री (लगभग २०.५३% और अधिकतम β -कैरोटीन सामग्री (१३०५ माइक्रोग्राम/किलोग्राम) था, जबकि बीएसएएलबी बीज के नमूनों में तेल की उपज लगभग २५.६४% थी। γ -टोकोफेरॉल उच्चतमपायागया, इसके बाद सभी बीज नमूनों में δ -टोकोफेरॉल और α -टोकोफेरॉल था। तेल में पांच प्रमुख फैटी एसिड होते हैं: मिथाइल पामिटेट (९.७९ से १०.८७%), मिथाइल स्टीरैट (३.१२ से ४.२६%), सीआईएस ९-ओलिक एसिड मिथाइल

एस्टर (१९.४६ से २५.४%), मिथाइल लिनोलेट (५४.६३ से ५७.४४%) और, मिथाइल लिनोलेनेट (५.८१ से ९.७५%) गैर-महत्व वितरण के साथ पाए गए। जीसी-एमएस/एचएस विश्लेषण से पहचाने गए लिपिड अंशों ने ४६ वाष्पशील स्वाद यौगिकों (वीएफसी) की सूचना दी। व्यक्तिगत लिपिड अंशों के बीच इन यौगिकों की संरचना में व्यापक अंतर था। सुपरक्रिटिकल फ्लुइड एक्सट्रैक्शन (एसएफई) ने १९.१% तेल की सूचना दी, जबकि सॉक्सलेट ने २३.६७% तेल उपज का उत्पादन किया। एसएफई तकनीक में असैपोनिफाइड पदार्थ और कैरोटीनॉयड सामग्री सॉक्सलेट निष्कर्षण (२.५७% और १.१३९ मिलीग्राम/किलोग्राम) की तुलना में थोड़ा अधिक (२.७३% और १.२५९ मिलीग्राम/किलोग्राम) थी, जो दर्शाता है कि एसएफई तेल तुलनात्मक रूप से उच्च एंटीऑक्सीडेंट गतिविधियों का प्रदर्शन करता है। एसएफई में फ्लेवर १,२-प्रोपेनडीओल, ३-बेंजाइलॉक्सी-१,२-डायसेटाइल- उच्च मात्रा में देखा गया।

१२ और ७२ घंटे के लिए काले सोयाबीन के नमूने (बीएसएनडीडी) को भिगोने से फाइटिक एसिड और रैफिनोज क्रमशः २८.९८% और ७४.५% तक कम हो गए। पारंपरिक मिट्टी के बर्तन और प्रेशर बरतन कुकिंग तकनीकों में क्रमशः ३२.५०% और ७५.०% तक फाइटिक और रैफिनोज कम हुआ, जबकि साइनाइडिन-३-ग्लूकोसाइड सभी उपचारों में काफी कम अपक्षीणन हो गया था। काले सोयाबीन से बने खाद्य उत्पाद के लिए उपभोक्ताओं के दृष्टिकोण को महसूस करने के लिए काले सोयाबीन के आटे पर आधारित लस मुक्त कुकीज़ तैयार की गई थी। स्वाद और रंग के लिए औसत संवेदी स्कोर ७.८ और ८.२ थे। ९-पॉइंट हेडोनिक स्केल पर बनावट, स्वाद और माउथफिल क्रमशः ८.१, ८.० और ८.१ थे और अच्छी उपभोक्ता स्वीकार्यता दिखाई।

अंत में, वर्तमान अध्ययन फाइटोकेमिकल और लिपिड संरचना के लिए स्थानीय काले सोयाबीन की वैज्ञानिक मान्यता को रिपोर्ट करता है। यह प्रोफाइलिंग स्थानीय और वैश्विक बाजारों में कम उपयोग वाले काले सोयाबीन के इस्तेमाल से विभिन्न मूल्य वर्धित और प्रोटीन युक्त पौष्टिक खाद्य उत्पादों को विकसित करने में मदद करेगी।

कुंजी शब्द: एंटीन्यूट्रिएंट्स; काले सोयाबीन, प्रीट्रीटमेंट; लिपिड विशेषताएँ; पोषण विरोधी कारक; गुणवत्ता विशेषताएँ; अल्ट्रासाउंड सहायता निष्कर्षण।

TABLE OF CONTENT

		Page No
	Certificate	<i>i</i>
	Acknowledgements	<i>ii-iii</i>
	Abstract	<i>iv-viii</i>
	List of Figures	<i>xv-xix</i>
	List of Tables	<i>xx-xxi</i>
	List of Symbols and Abbreviations	<i>xxii-xxiv</i>
Chapter I	Introduction	1-4
Chapter II	Literature Review	5-18
2.1	<i>Black Soybean Overview</i>	5
2.1.1	Botanical description of <i>Black Soybean (Glycine Max (L). Merrill)</i>	5
2.1.2	Global and local production scenario of soybean	5
2.1.3	<i>Black soybean vs yellow soybean</i>	7
2.1.4	Phytonutrients in <i>black soybean</i>	7
2.1.5	Therapeutic role of phytonutrients presents in <i>black soybean</i>	9
2.2	Extraction and Identification of Phytochemicals	10
2.2.1	Solvent extraction	10
2.2.2	Ultrasonic assisted extraction	11
2.2.3	Microwave assisted extraction	11
2.2.4	Identification and quantification of phytochemicals	11
2.3	Lipid Extraction and Characterization	13
2.3.1	Mechanical extraction	13
2.3.2	Soxhlet extraction	13
2.3.3	Ultrasonic assisted extraction	13
2.3.4	Microwave assisted extraction	14
2.3.5	Supercritical fluid extraction	14
2.3.6	Lipid characterization	14
2.4	Antinutritional Factors	15

2.4.1	Phytic acid content	15
2.4.2	Raffinose content	15
2.4.2	Trypsin inhibitor	16
2.5	Effect of Processing on Isoflavones and Anthocyanins	16
2.6	Effect of Processing on Anti-Nutritional Factors	17
2.7	Availability of <i>Black Soybean</i> -Based Products	17
2.8	Research Gaps	18
Chapter III	Characterization of the Phytochemical Properties of <i>Black Soybean</i> Produced in Indo-Himalayan Regions	19-65
3.1	Material and methods	19
3.1.1	Chemicals and reagents	19
3.1.2	Sample collection	20
3.1.3	Physico-chemical characteristics measurement	20
3.1.3.1	Seed measurement	20
3.1.3.2	Proximate analysis	21
3.1.3.3	Sucrose analysis by HPLC-RID	22
3.1.3.4	Elemental analysis by CHNS analyzer	22
3.1.4	Fourier Transform Infrared (FTIR) Spectrometric identification	22
3.1.5	Amino acid analysis	22
3.1.5.1	Protein hydrolysis	22
3.1.5.2	Sample pre-column derivatization	23
3.1.5.3	Preparation of standard curve	23
3.1.5.4	HPLC separation	24
3.1.6	Mineral analysis	25
3.1.6.1	Sample digestion	25
3.1.6.2	Standard curve	25
3.1.6.3	Mineral estimation by ICPOES	25
3.1.7	DPPH antioxidant assay	26
3.1.8	Total phenolic content	26
3.1.9	Total flavonoid content	26

3.1.10	UAE extraction of polyphenols from <i>black soybean</i> samples	27
3.1.10.1	Experimental design and statistical analysis	27
3.1.10.2	Ultrasound-assisted extraction experiments	28
3.1.11	Isoflavones estimation	28
3.1.12	Anthocyanin (Cyanidin-3 glucoside) estimation	29
3.1.13	Polyphenol estimation by HPLC-UV	29
3.1.14	Bioactive screening in the methanolic extract	30
3.1.15	Statistical analysis	31
3.2	Results and Discussion	31
3.2.1	Physico-chemical properties of <i>black soybean</i> samples	31
3.2.1.1	Seed dimensions	31
3.2.1.2	Proximate composition	32
3.2.1.3	Sucrose composition	33
3.2.2	Functional characterization of the seed extract	34
3.2.3	Protein quality assessment by individual amino acids	35
3.2.4	Macro and micronutrients characteristics	39
3.2.5	Principal component analysis between amino acid, micronutrients, proximate composition, and seed dimensions	43
3.2.6	Optimization of isoflavone/anthocyanin extraction	44
3.2.6.1	Preliminary optimization of extraction methodology	44
3.2.6.2	UAE experiment design matrix	47
3.2.6.3	Model suitability	47
3.2.7	DPPH assay, TPC and TFC of extract at optimum UAE conditions	53
3.2.8	β -glucoside and aglycon isoflavone composition	54
3.2.9	Cyanidin-3-glucoside anthocyanin analysis	59
3.2.10	Polyphenol estimation in <i>black soybean</i>	62
3.2.11	Bioactive screening in the methanolic extract	63
3.3	Conclusion	64

Chapter IV	Analysis of the Lipid Profile of <i>Black Soybean</i> Oil for Fatty Acids, Flavour, and Antioxidants	66-88
4.1	Material and Methods	67
4.1.1	Chemical and reagents	67
4.1.2	Sample details	67
4.1.3	Oil extraction	68
4.1.3.1	Soxhlet extraction	68
4.1.3.2	Supercritical fluid extraction	68
4.1.4	FTIR analysis	68
4.1.5	β -carotene estimation by UV-VIS method	68
4.1.6	Unsaponifiable matter	69
4.1.7	Tocopherol analysis	69
4.1.8	Fatty acid analysis by GC	70
4.1.9	Flavor and other volatiles by GCMS-HS	70
4.1.10	Statistical analysis	71
4.2	Results and Discussion	71
4.2.1	Oil yield optimization	71
4.2.2	FTIR analysis	72
4.2.3	β -carotene properties	73
4.2.4	Unsaponifiable properties	74
4.2.5	Tocopherol characteristics	75
4.2.6	Fatty acid characterization in the extracted oil	76
4.2.7	Flavor and other volatile properties in the extracted oil	80
4.2.8	Comparison between Soxhlet and supercritical fluid extracted oil	86
4.3	Conclusion	88
Chapter V	Investigation of the Effect of Treatments viz. Pressure, Microwave, and Conventional Cooking Methods on Reducing the Antinutritional Factors of <i>Black Soybean</i>	89-102

5.1	Material and Methods	90
5.1.1	Samples	90
5.1.2	Soaking	90
5.1.3	Microwave cooking	90
5.1.4	Pressure cooking	91
5.1.5	Conventional clay pot cooking	91
5.1.6	Phytic acid by colorimetric method	91
5.1.7	Raffinose analysis by HPLC-RID	92
5.1.8	Statistical analysis	93
5.2	Results and Discussion	93
5.2.1	Phytic and Raffinose content in different <i>black soybean</i> seeds	93
5.2.2	Effect of treatment on phytic acid	94
5.2.2.1	Soaking	94
5.2.2.2	Microwave treatment	95
5.2.2.3	Pressure cooking	96
5.2.2.4	Conventional cooking	96
5.2.3	Effect of cooking methods on raffinose content	97
5.2.3.1	Soaking	98
5.2.3.2	Microwave cooking	99
5.2.3.3	Pressure cooking	100
5.2.3.4	Conventional cooking	100
5.2.4	Effect of treatment on anthocyanin (cyanidin-3-glucoside) content	101
5.3	Conclusion	102
Chapter VI	To Develop <i>Black Soybean</i> Based Value-Added Food Product and Analysis of Quality Attributes and Consumer Perceptions	103-113
6.1	Material and Methods	104
6.1.1	Cookies preparation	104
6.1.2	Quality analysis of the cookies	104
6.1.2.1	Proximate composition	104

6.1.2.2	DPPH antioxidant assay	105
6.1.3	Mineral analysis	105
6.1.4	Texture profile analysis	105
6.1.5	Sensory evaluation	105
6.2	Results and Discussion	105
6.2.1	Cookies preparation flow diagram	105
6.2.2	Proximate composition and quality evaluation	107
6.2.3	Mineral composition	109
6.2.4	Texture analysis	109
6.2.5	Polyphenol estimation	110
6.2.6	Sensory evaluation	110
6.2.7	Cost estimation of the developed cookies	112
6.3	Conclusion	113
Chapter VII	Overall Summary and Conclusion	114-125
	References	126-138
	Appendix	139-150

LIST OF FIGURES

Figure	Description	Page No.
2.1	Pictorial views of <i>black soybean</i> (a) and (b) plant, (c) pods and (d) seeds	6
2.2	Global soybean production data (2022-23)	6
3.1	Overview of the nutritional parameters characterized in this chapter	20
3.2	<i>Black soybean</i> samples collected from Indo-Himalayan regions: (a) is BSNDD from Dehradun, (b) BSRMN from Ramnagar, (c) BSALB from Almora, (d) BSGRP from Nainital and (e) is BSALS from Almora. (a) and (c) are big seeds, (b) and (d) are medium shape seeds and (e) is small shaped seeds	21
3.3	Effect of evaporation after sample hydrolysis on the individual amino acid peak intensity	23
3.4	Effect of mobile phase on the individual amino acid peak separation	24
3.5	Standard and sample carbohydrate separation HPLC chromatogram	34
3.6	Comparative samples and isoflavone standard FTIR spectra	35
3.7	Seventeen amino acid standard mixture representative HPLC chromatogram	36
3.8	Amino acid distribution among different <i>black soybean</i> samples. * Indicates essential amino acids	37
3.9	Stacked standard and sample amino acid separation chromatograms of five <i>black soybean</i> samples	38
3.10	Representative Na and Mn peak spectra for black soybean samples obtained from ICP-OES	41
3.11	Micronutrients distribution in different <i>black soybean</i> samples	41
3.12	Representative calcium standard and sample ICP-OES spectra at 317.9 nm	42

3.13	Macronutrients distribution in different <i>black soybean</i> sample	43
3.14	PCA bi-plot of two principal components for different quality characteristics of <i>black soybean</i>	44
3.15	Black soybean seeds treatment in a different medium. (a) Sodium bicarbonate, (b) Sodium hydroxide, (c) Sodium chloride, (d) Citric acid, (e) Hydrochloric acid, (f) Acetic acid, (g) Hydrogen peroxide, and (h) control without treatment	45
3.16	Cyanidin-3-glucoside (C3G) detection pattern in different medium	45
3.17	Effect of various extraction methods on three major FTIR peaks at 1015, 1640 and, 2835 cm ⁻¹ wavenumbers. UAE-Ultrasonic assisted extraction; LS-Liquid sonication, and SH-Water bath shaking	46
3.18	Effect of various extraction methods on glucoside isoflavones Effect of various extraction methods on β -glucoside isoflavones. UAE-Ultrasonic assisted extraction; LS-Liquid sonication, SH-Water bath shaking	46
3.19	Interaction effect of solvent/solid ratio versus time (a) and solvent/solid ratio verses amplitude (b) on DPPH content	51
3.20	Interaction effect of (a) amplitude and ultrasound time and (b) solvent-to-solid ratio and ultrasound amplitude on TPC yield	52
3.21	Interaction effect of (a) ultrasound time and solvent-to-solid ratio and (b) ultrasound amplitude and time on TFC content	52
3.22	Theoretical validation of actual and predicted values of DPPH assay, TPC and TFC of <i>black soybean</i> upon UAE at optimum conditions	53
3.23	Flow chart for the extraction of anthocyanin and isoflavones	55

3.24	Isoflavones HPLC chromatogram of five <i>black soybean</i> samples. (a) BSALS, (b) BSALB, (c) BSNDD, (d) BSRMN and (e) BSGRP	57
3.25	Cyandin-3-glucoside HPLC peak distribution in five <i>black soybean</i> samples; (a) BSALS, (b) BSALB, (c) BSNDD, (d) BSRMN and BSGRP seed samples	61
3.26	Representative HPLC chromatogram of polyphenol analysis (Gallic at 2.916 min, Caffeic at 4.139 min and Quercetin at 14.278 min RT)	62
3.27	Representative total ion chromatogram of methanolic extract in the GCMS analysis	64
4.1	Overview of the phytoconstituents studied in the lipid extract of <i>black soybean</i> samples	67
4.2	Representative thirty-seven fatty acids standard GC chromatogram	71
4.3	Five black soybean lipid extract FTIR spectrum showing the peaks at 2958 cm ⁻¹ , 2924 cm ⁻¹ , 2860 cm ⁻¹ , 1751 cm ⁻¹ , 1462 cm ⁻¹ , 1378 cm ⁻¹ , and 724 cm ⁻¹ wavenumbers	73
4.4	β-carotene and total tocopherol comparison in different oil extract of <i>black soybean</i> seed sample	74
4.5	Unsaponifiable matter after drying of ether extract in BSALS and BSNDD evaporated at 80-90 °C	74
4.6	Representative normal phase HPLC chromatogram showing tocopherol separation	76
4.7	Fatty acid comparison in different oil extract. BSALS and BSALB: <i>Black soybean</i> seeds from Almora; BSNDD from Dehradun; BSRMN from Ramnagar; BSGRP from Nainital	77
4.8	Individual sample chromatogram showing the fatty acid separation in the oil extract. BSALS and BSALB: <i>Black soybean</i> seeds from Almora; BSNDD from Dehradun; BSRMN from Ramnagar; BSGRP from Nainital	79

4.9	Flavor/volatile component distribution in five individual BS oil extracts (a), Pie diagram showing the overall 46 flavor distribution in BS oil extract (b)	80
4.10	GCMS-HS flavor profile total ion chromatogram of the individual samples	84
4.11	Fatty acid comparison of oil extracted Soxhlet and Supercritical CO ₂ extraction by a SCF techniques	87
5.1	Overview of different cooking methods designed to observe the effect on anti-nutritional factors	90
5.2	Phytic acid reverse standard linearity curve prepared for phytic estimation by colorimetry	92
5.3	Effect of soaking on phytic acid reduction in <i>black soybean</i>	95
5.4	Effect of microwave cooking on phytic acid reduction (P60, 80 and 100 corresponds to 400, 800 and 1200 W microwave treatment for 5, 10 and 15 min, respectively)	95
5.5	Effect of pressure cooking on phytic acid reduction of black soybean	96
5.6	Effect of conventional cooking on phytic acid in <i>black soybean</i>	97
5.7	Representative raffinose standard HPLC-RID chromatogram	98
5.8	Effect of soaking on raffinose reduction	99
5.9	Effect of microwave cooking on raffinose reduction (P60, 80 and 100 corresponds to 400, 800 and 1200 W microwave treatment for 5, 10 and 15 min, respectively) in <i>black soybean</i> seeds	99
5.10	Effect of pressure vessel cooking (PC) on raffinose reduction in black soybean seeds	100
5.11	Effect of conventional cooking on raffinose reduction in <i>black soybean</i> seeds	101
6.1	Overview of the quality evaluation of the developed product	104
6.2	Process flow diagram for the preparation of soybean-based cookies	106

6.3	Images of black soybean and jaggery based cookies	107
6.4	Sensory evaluation analysis of <i>black soybean</i> based cookies on a 9-point hedonic score	111
6.5	Cookies cost comparison with the market products	112

LIST OF TABLES

Table no.	Description	Page No.
2.1	Comparison between yellow and <i>black soybean</i>	8
3.1	Physical dimensions of the <i>black soybean</i> samples	31
3.2	Proximate composition of five <i>black soybean</i> samples	32
3.3	Sucrose content of five <i>black soybean</i> seed samples	33
3.4	Amino acid (AA) composition in <i>black soybean</i> seed samples	35
3.5	Mineral composition of <i>black soybean</i> seeds	40
3.6	Experimental variables and their levels for UAE extraction	47
3.7	Experimental design of UAE variables and their responses (DPPH, TPC and TFC)	48
3.8	CCD matrix of three variables with experimental and predicted values of process parameters at optimized condition	50
3.9	Analysis of variance (ANOVA) for the fit of independent factors to the response surface model	50
3.10	DPPH assay, TPC and TFC profiling of <i>black soybean</i> at optimum UAE conditions	54
3.11	Isoflavones estimation in <i>black soybean</i> seeds by HPLC-UV	58
3.12	Cyanidin-3-glucoside content of <i>black soybean</i> samples by HPLC-UV	60
3.13	Polyphenol analysis in <i>black soybean</i> seeds by HPLC	62
3.14	Major bio-actives identified in the methanolic extract by GCMS	63
4.1	Yield, unsaponified matter, β -Carotene and tocopherol composition in lipid extract	72
4.2	Individual tocopherol composition in five <i>black soybean</i> samples	75
4.3	Fatty acid compositions of five <i>black soybean</i> samples	77
4.4	Flavor and other volatile profiling in the lipid extracts of five <i>black soybean</i> seeds	84

4.5	Lipid quality comparison between the Soxhlet and Supercritical CO ₂ extraction	87
4.6	Comparison between SFE and Soxhlet extracted oil in volatile components	88
5.1	Phytic acid content in different <i>black soybean</i> samples	93
5.2	Raffinose content in different <i>black soybean</i> samples	93
5.3	Phytic acid content in different cooking treatments	94
5.4	Raffinose content in <i>black soybean</i> samples reduced by different cooking methods	98
5.5	Cyanidin-3-glucoside content in different cooking treatment Cyanidin-3-glucoside content in different cooking treatments	102
6.1	Proximate composition of the <i>black soybean</i> cookies	107
6.2	Percent area amino acid (AA) composition in the cookie's protein	108
6.3	Assessment of <i>black soybean</i> cookies as per IS 1011 and FSSR-2011 specification	109
6.4	Cookies mineral composition	109
6.5	Cookies polyphenolic profile by HPLC-PDA	110
6.6	Sensory analysis of <i>black soybean</i> based cookies on 9-point hedonic scale	111
6.7	Cookies cost estimation per 400 g serving	113

LIST OF SYMBOLS AND ABBREVIATIONS

Syntax	Description
%	Percent
<	Less than
>	Greater than
@	At the rate
°C	Degree Celsius
g	gram
h	Hour
kg	Kilogram
L	Litre
t	Time
R ²	Co-efficient of determination
s	Second
µm	micro meter
µL	micro litre
v	volume
w	weight
W	Watt
AU	Absorbance unit
AA	Amino acid
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
BS	Black soybean
BSALS	Small oval shape seed from Almora
BSALB	Big spherical shape seed from Almora
BSNDD	Big spherical shape seed from Dehradun
BSRMN	Medium spherical shape seed from Ramnagar

BSGRP	Medium spherical shape seed from Nainital
CO ₂	Carbon Dioxide
C3G	Cyanidin-3-glucoside
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimeter
EPR	Electron Paramagnetic Resonance
EtOH	Ethyl alcohol
FAME	Fatty acid methyl esters
Fig.	Figure
FRAP	Ferric reducing-anti-oxidative power
FRSA	Free radical-scavenging activities
FSSAI	Food Safety and Standards Authority of India
FSSR	Food Safety and Standard Regulations
FTIR	Fourier Transform Infrared Spectrophotometer
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectroscopy
H ₂ O	Water
HHP	High hydrostatic pressure
HPLC	High Performance Liquid Chromatography
HS	Headspace
ICPOES	Inductive Coupled Plasma Optical Emission Spectrophotometer
IISR	Indian Institute of Soybean Research
IS	Indian Standard
ISO	International Organization for Standardization
InsP ₆	myoinositol hexaphosphate
kPa	Kilopascal
MAE	Microwave assisted extraction
min.	minute
mg	milligram
mm	milli meter

m/z	mass to charge ratio
mL	milli litre
NIST	National Institute of Standards and Technology
psi	Pounds per square inch
P	Phosphorous
PCA	Principal Component Analysis
PUFA	Polyunsaturated fatty acid
ppm	parts per million
RID	Refractive Index Detector
RSM	Response Surface Methodology
SD	Standard deviation
SFE	Supercritical fluid extraction
SSR	Solvent to solid ratio
TAC	Total anthocyanin content
TIC	Total isoflavone content
TPC	Total phenolic content
TFC	Total flavonoid content
UV	Ultraviolet
VIS	Visible
