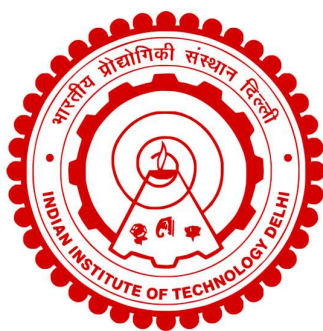


**EXTRACTION AND CHARACTERIZATION OF BIOACTIVES
FROM GILOY (*TINOSPORA CORDIFOLIA*) AND ITS
APPLICATION IN HERBAL YOGURT FORMULATION**

NITIN KUMAR



**CENTRE FOR RURAL DEVELOPMENT AND TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

JULY 2023

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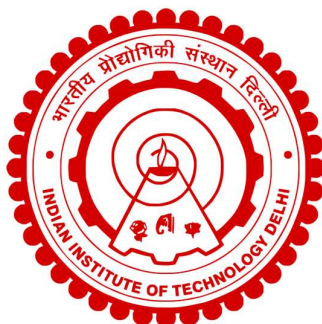
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CERTIFICATE

This is to certify that the thesis entitled “**Extraction and Characterization of Bioactives from Giloy (*Tinospora cordifolia*) and its Application in Herbal Yogurt Formulation**” being submitted by **Nitin 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 the thesis. To the best of our knowledge, the results contained in the thesis have not been submitted, in part or full, to any other university or institute for the award of any degree or diploma.

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Centre for Rural Development & Technology

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(Prof S N Naik)

Centre for Rural Development & Technology

Indian Institute of Technology Delhi

Date: 27th July, 2023

Place: New Delhi

Dedicated to

My Beloved Family

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ABSTRACT

Giloy (*Tinospora cordifolia*), a medicinal herb, traditionally used in complementary and alternate medicine owing to its various positive impact on human health i.e. antioxidant, anti-inflammatory, anti-allergenic, antidiabetic, and immunomodulatory characteristics. Almost all the parts of giloy plant such as leaves stems, and roots have potential biological benefits because of the presence of secondary metabolites such as alkaloids, diterpenoid lactones, terpenoids, lignans, steroids, and other phenolic compounds. Keeping the therapeutic potential of giloy extract for the holistic benefit in food, human nutrition and health, a process technology was aimed to develop a herbal yoghurt using giloy stem extract (GSE) as a natural antioxidant. To achieve this, the present research work was comprised of extraction of GSE using microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), thereafter identification of bioactive compounds using Gas chromatography–mass spectrometry (GC-MS), and High resolution-liquid chromatography mass spectrometry-quadrupole-time-of-flight (HR-LCMS-QToF) techniques. The optimized GSE was further utilized for the formulation of herbal functional yoghurt followed by the evaluation of shelf life under refrigerated storage conditions.

A Box–Behnken design of response surface methodology (RSM) was employed for the optimization of yield, total phenolic content (TPC) and antioxidant activity (1,1-diphenyl-2-picrylhydrazyl, (DPPH)) of GSE with solvent-to-solid ratio (SS ratio) and MAE variables (extraction time, microwave power) and UAE variables (extraction time and ultrasound (US) amplitude). The SS ratio, microwave power, US amplitude, and extraction time exhibited an individual effect on the total yield, TPC and DPPH values of the GSE. However, the US amplitude and extraction time had the most significant ($p < 0.05$) effect on the GSE yield. The MAE was observed to produce a higher GSE yield however, the UAE-assisted GSE was found to have better phenolic and antioxidant values. Based on the numerical optimization, the optimum process conditions under UAE were found to be 30% US amplitude, 10:1 SS ratio and 30 min extraction time. The optimum values of the extract yield: $9.614 \pm 0.015\%$, TPC: 100.04 ± 0.133 mg GAE per 100g extract, and DPPH inhibition: $78.8523 \pm 0.399\%$ with the percentage of the error

between the predicted and experimental values less than 5%. Screening of optimized extract through GC-MS, and HR-LCMS-QTOF analysis detected the presence of several polyphenolic compounds such as 1,2-oxathiane, 6-dodecyl-, 2,2-dioxide, silanol, trimethyl-, phosphate (3:1), 2-propenoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester, methyl 9,10-octadecadienoate, (-)-catechin quercitrin, malic acid and aurasperone E among others.

Development of GSE-based herbal yoghurt was formulated using a central composite rotatable design of RSM with GSE concentration, incubation temperature (IT), and total soluble solid (TSS) as independent variables and whiteness index, overall acceptability, pH, syneresis, firmness, DPPH scavenging activity, and complex viscosity were measured as response variables. The results revealed that GSE and TSS exhibited maximum effects on all the responses. The optimum formulation condition was found to be 0.08% GSE, 45°C IT, and 9.1% TSS where whiteness index, overall acceptability, pH, syneresis, firmness, DPPH radical scavenging activity and complex viscosity were predicted to be 84.513%, 8.567, 4.193, 47.268%, 281.190 N, 56.78% and 76.89 cP respectively. The oscillatory rheology attributed a viscoelastic behaviour in the yoghurt which was significantly modulated by the addition of GSE.

Storage qualities of the yoghurt sample were assessed for 21 days under the refrigerated storage condition ($4\pm1^{\circ}\text{C}$). Both the control (without the addition of GSE) and herbal yoghurt exhibited a shear-thinning behaviour during the storage. The herbal yoghurt had significantly greater ($p < 0.05$) DPPH activity compared to the control. The fat and protein in yoghurt were observed to decrease with the storage period. The herbal yoghurt showed significant α -analyses and α -glucosidase inhibition activity in comparison to control. The developed herbal yoghurt was found to have a shelf-life of 21 days under refrigerated storage ($4\pm1^{\circ}\text{C}$) with all the quality and sensory attributes acceptable. The estimated cost per 200 mL herbal yoghurt cup was calculated to be ₹ 23.50 with a 20% profit margin.

Keywords: *Giloy; Green extract; Bioactive compounds; Ultrasound-assisted extraction; Herbal yoghurt; Antidiabetic properties; Shelf life*

अमूर्त

गिलोय (*टीनोस्पोरा कार्डिफ़ोलिया*) एक औषधीय जड़ी बूटी है। जिसे पारंपरिक रूप से पूरक और वैकल्पिक चिकित्सा में मानव स्वास्थ्य पर सकारात्मक प्रभाव के कारण उपयोग की जाती है। गिलोय के अंदर बहुत सारी औषधीय गुण होते हैं, जैसे कि एंटीऑक्सिडेंट, एंटी-इंफ्लेमेटरी, एंटी-एलर्जेनिक, एंटीडायबिटिक और इम्यूनोमॉड्यूलेटरी। गिलोय के पौधे के लगभग सभी भागों जैसे पत्तियां, तने और जड़ों में कई माध्यमिक मेटाबोलाइट्स जैसे अल्कलॉइड्स, डाइटरपेनॉइड लैक्टोन्स, टेरपेनोइड्स, लिग्नन्स, स्टेरॉयड और अन्य फेनोलिक यौगिकों की उपस्थिति के कारण संभावित जैविक लाभ प्राप्त होते हैं। इसलिए, वर्तमान शोध कार्य में माइक्रोवेव असिस्टेड एक्सट्रैक्शन और अल्ट्रासाउंड असिस्टेड एक्सट्रैक्शन का उपयोग करके गिलोय के तने से अर्क का एक्सट्रैक्शन शामिल है, इसके उपरांत क्रोमैटोग्राफी-मास स्पेक्ट्रोमेट्री और एचआर-एलसीएमएस-क्यूटीओएफ का उपयोग करके अर्क में मौजूद बायोएक्टिव की पहचान की जा सके। अनुकूलित अर्क का हर्बल योगर्ट के निर्माण के लिए उपयोग किया गया और इसकी शेल्फ लाइफ का मूल्यांकन प्रशीतित स्थिति में किया गया था।

विलायक-से-ठोस अनुपात, एक्सट्रैक्शन टाइम और माइक्रोवेव पावर या अल्ट्रासाउंड आयाम के संबंध में जी.एस.ई की उपज, कुल फेनोलिक सामग्री और एंटीऑक्सीडेंट गतिविधि के अनुकूलन के लिए आर.एस.एम के बॉक्स-बेहेनकेन डिजाइन को नियोजित किया गया था। विलायक-से-ठोस अनुपात, माइक्रोवेव पावर, अल्ट्रासोनिक आयाम और एक्सट्रैक्शन टाइम ने लगभग सभी प्रतिक्रियाओं पर अपनी व्यक्तिगत प्रभाव प्रदर्शित किया। हालांकि, अल्ट्रासाउंड पावर आयाम और सोनिकेशन टाइम का जी.एस.ई के उत्पादन पर सबसे महत्वपूर्ण प्रभाव पड़ा। कुल मिलाकर, माइक्रोवेव असिस्टेड एक्सट्रैक्शन के परिणामस्वरूप ज्यादा मात्रा में जी.एस.ई मिला, लेकिन, अल्ट्रासाउंड असिस्टेड एक्सट्रैक्शन की सहायता से बेहतर गुणवत्ता वाली जी.एस.ई पाई गई। संख्यात्मक अनुकूलन के आधार पर, अल्ट्रासाउंड असिस्टेड एक्सट्रैक्शन के तहत इष्टतम प्रक्रिया की स्थिति: 30% अल्ट्रासाउंड आयाम, 10:1 विलायक-से-ठोस अनुपात और 30 मिनट सोनिकेशन टाइम के रूप में पाई गई। इष्टतम प्रक्रिया के स्थितियों में अर्क: $9.614 \pm 0.015\%$, टी.पी.सी: 100.04 ± 0.133 (मिलीग्राम जी.एस.ई प्रति 100 ग्राम अर्क), और डी.पी.पी.एच% निषेध: $78.8523 \pm 0.399\%$ शामिल है जहाँ कि इष्टतम अनुमानित महत्व और प्रयोगात्मक महत्व के बीच त्रुटि 5 प्रतिशत से भी कम था। जीसी-एमएस, और एचआर-एलसीएमएस-क्यूटीओएफ के माध्यम से अनुकूलित अर्क की स्क्रीनिंग ने कई पॉलीफेनोलिक यौगिकों जैसे 1,2-ऑक्साथियान, 6-डोडेसिल-, 2,2-डाइऑक्साइड, सिलानोल, ट्राइमिथाइल-, फॉस्फेट (3:1), 1,2-प्रोपेनोइक एसिड, 2-[ट्राइमिथाइलसिलिल) ऑक्सी]-, ट्राइमेथिलसिलिल एस्टर, मिथाइल 9,10-ऑक्टाडेकेडीनोएट, (-)- कैटेचिन

केरसिट्रिन, मैलिक एसिड, और ऑरॉस्पेरोन ई, आदि की उपस्थिति की सूचना दी। अतएव यह साबित होता है कि गिलोय के अर्क में एंटीऑक्सिडेंट, पॉलीफेनोल, फ्लेवोनोइड्स आदि जैसे न्यूट्रास्यूटिकल्स का अच्छा समावेश है और यह फंक्शनल खाद्य पदार्थों के विकास के लिए एक उत्कृष्ट घटक साबित हो सकता है।

योगर्ट एक प्रमुख किण्वित दूध उत्पाद है और गिलोय के औषधीय लाभों को शामिल करने के लिए यह एक खाद्य मैट्रिक्स वाहक के रूप में कार्य करेगा। इसलिए, रिस्पांस सरफेस कार्यप्रणाली के एक केंद्रीय समग्र रोटेटेबल डिज़ाइन (CCRD) के माध्यम से जी.एस.ई आधारित हर्बल योगर्ट के विकास और अनुकूलन को जी.एस.ई एकाग्रता, ऊष्मायन तापमान, और कुल घुलनशील ठोस के साथ एक स्वतंत्र पैरामीटर के रूप में खोजा गया, जबकि सफेदी सूचकांक, समग्र स्वीकार्यता, पीएच, तालमेल, दृढ़ता, डीपीपीएच की गतिविधि और जटिल चिपचिपाहट को प्रतिक्रियाओं के रूप में माना गया। परिणामों से पता चला कि जी.एस.ई और टी.एस.एस ने सभी प्रतिक्रियाओं पर अधिकतम प्रभाव प्रदर्शित किया। संख्यात्मक अनुकूलन के आधार पर, इष्टतम सूत्रीकरण की स्थिति: 0.08% जी.एस.ई, 45°C ऊष्मायन तापमान, और 9.1% कुल घुलनशील ठोस पाई गई। प्रतिक्रियाओं की इष्टतम स्थिति इस प्रकार थी- श्वेतता सूचकांक: 84.513%, समग्र स्वीकार्यता स्कोर: 8.567, पीएच: 4.193, साइनेरेसिस: 47.268%, दृढ़ता: 281.190N, डी.पी.पी.एच रेडिकल स्कैवेंजिंग गतिविधि: 56.78%, और जटिल चिपचिपाहट 76.89cp। ऑसिलेटरी रियोलॉजी ने योगर्ट में एक विस्कोलेस्टिक व्यवहार को जिम्मेदार ठहराया जो कि जी.एस.ई के मिलाने द्वारा संशोधित किया गया था। अनुमानित महत्व और प्रयोगात्मक महत्व के बीच की त्रुटि सांख्यिकीय रूप से ($p < 0.05$) महत्वपूर्ण नहीं थी।

योगर्ट के नमूने का मूल्यांकन 21 दिनों के भंडारण अवधि तक किया गया और नियंत्रण और हर्बल योगर्ट दोनों ने शिअर थिनिंग जैसा व्यवहार प्रदर्शित किया। प्रशीतित भंडारण अवधि में नियंत्रित योगर्ट की तुलना में हर्बल योगर्ट में काफी अधिक ($p < 0.05$) एंटीऑक्सीडेंट गतिविधि थी। यह पाया गया कि, योगर्ट में मौजूद वसा और प्रोटीन भंडारण अवधि के साथ घट रहे हैं, जिसका कारण लिपोलाइसिस और प्रोटियोलिटिक परिवर्तन हो सकते हैं। हर्बल योगर्ट की पॉलीफेनोलिक सामग्री और एंटीडायबिटिक गतिविधि उपभोक्ताओं के लिए एक अतिरिक्त लाभ है। यह देखा गया कि जी.एस.ई समृद्ध हर्बल योगर्ट में कैल्शियम की मात्रा अन्य योगर्ट की तुलना में अधिक थी। विकसित हर्बल योगर्ट में सार्थक α -एमाइलेज और α -ग्लूकोसिडेस निषेध दिखाई दिया, जो इसके एंटीडायबिटिक गुणों का संकेत देता है। प्रशीतित भंडारण के तहत विकसित हर्बल योगर्ट में सभी गुणवत्ता और संवेदी विशेषताओं के साथ 21 दिनों का शेल्फ जीवन था। 20% लाभ मार्जिन के साथ प्रति 200 एमएल हर्बल योगर्ट कप की अनुमानित लागत 23.50 रुपये थी।

मुख्य शब्द: गिलोय, अर्क, कुल फेनोलिक सामग्री, अल्ट्रासाउंड असिस्टेड एक्सट्रैक्शन, अनुकूलन, बायोएक्टिव्स, हर्बल योगर्ट, एंटीडायबिटिक गुण और शेल्फ लाइफ

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LIST OF SYMBOLS AND ABBREVIATIONS

α	alpha
%	Percent
pH	Potential of Hydrogen
$\pm$	plus-minus sign
<	Less than
>	Greater than
$\geq$	Greater than or equal to
@	At the rate
USD	United States Dollar
₹	Indian Rupee
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
DAHD	Department of Animal Husbandry & Dairying
FSSAI	Food Safety and Standards Authority of India
USFDA	United State Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
EFSA	European Food Safety Authority
NDRI	National Dairy Research Institute
NCDC	National Collection of Dairy Cultures
FMPs	Fermented milk products
MAE	Microwave assisted extraction
UAE	Ultrasound assisted extraction
SCFE	Supercritical fluid extraction
DESE	Deep eutectic solvent extraction
PEF	Pulsed electric field
max.	Maximum
min.	Minute
Ca	Calcium
Cr	Chromium
Fe	Iron
Zn	Zinc

P	Phosphorus
K	Potassium
Na	Sodium
°C	Degree Celsius
viz.	Videlicet (namely)
Eq.	Equation
Fig.	Figure
FTIR	Fourier transform infrared
g	gram
g.L ⁻¹	gram per litre
G'	Storage modulus
G''	Loss modulus
η*	Complex viscosity
G*	Complex modulus
GCMS	Gas Chromatography Mass Spectroscopy
LCMS	Liquid Chromatography Mass Spectroscopy
h	Hour
HPLC	High Performance Liquid Chromatography
GCTM	Global Centre for Traditional Medicine
ISO	International Organization for Standardization
WHO	World Health Organization
kcal	kilocalorie
kg	kilogram
kg.m ⁻³	kilogram per meter cube
kg. s ⁻¹	kilogram per second
LAB	Lactic acid bacteria
w	weight
wb	wet basis
db	dry basis
τ	shear stress
γ	shear rate
cfu. mL ⁻¹	Colony forming unit per mili Litre
μm	micrometer

μL	micro Litre
mbar	milibar
cm	centimeter
mm	milimeter
m/z	mass to charge ratio
min	minute
meq	mili equivalent
mg	mili gram
mg QE. g^{-1}	milligram quercetin equivalents per gram
Mg GAE. g^{-1}	milligram gallic acid equivalents per gram
AC	Alternating current
mL	mili Litre
$\text{mL}.\text{g}^{-1}$	mili Litre per gram
Hz	Hertz
MHz	Mega Hertz
$^{\circ}\text{C}$	Degree Celsius
Pa	Pascal
MPa	mega Pascal
cP	centipoise
Pa.s	Pascal second
R^2	Co-efficient of determination
rpm	Revolution per minute
rpm	Revolution per second
$\text{rad}.\text{s}^{-1}$	Radian per second
s	Second
v	volume
