Phytochemical Screening, Quantification, FT-IR Analysis, and *In Silico* Characterization of Potential Bio-active Compounds Identified in HR-LC/MS Analysis of the Polyherbal Formulation from Northeast India

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ABSTRACT: Diabetes is a group of metabolic disorders characterized by elevated blood sugar levels, leading to many undesirable health consequences. There are many herbal formulations, traditionally used by the Northeast Indian population for disease management. These formulations require scientific validations to optimize their efficacy and increase their popularity. In this study, we attempt to scientifically validate a polyherbal formulation traditionally used for the management of diabetes through preliminary phytochemicals investigation, characterization of potential phytochemicals using Fourier transform infrared (FT-IR) spectroscopy, high-resolution liquid chromatography mass spectrometry (HR-LC/MS) analysis, and *in silico* characterization of physiochemical, drug-likeness, and pharmacokinetic properties of identified phytochemical compounds. Qualitative phytochemical screening of various extracts of the formulation confirmed the presence of alkaloids, phenols and tannins, flavonoids, fats, and oils. Phytochemical quantification of the various extracts showed that the highest total phenolic content is present in the ethanolic extract (35.61 ± 0.15 mg GAE/g), while the highest total flavonoid content is present in the chloroform extract (76.33 ± 2.96 mg QE/g) of the formulation. FT-IR spectroscopic analysis revealed various characteristic band values with various functional groups in the formulation extract such as amines, alcohol, fluoro compounds, phenol, alkane, alkene, and conjugated acid groups. HR-LC/MS analyses identified nearly 51 compounds including 9 small peptides and 42 potential phytochemical compounds. *In silico* SwissADME analysis of identified compounds revealed 25 potential compounds following Lipinski's rule and showing drug-like characteristics, and out of them, 16 compounds exhibited good oral bioavailability, as revealed in the bioavailability radar. The overall study showed that the presented polyherbal formulation is enriched with bio-active phytochemical compounds with good pharmaceutical values.
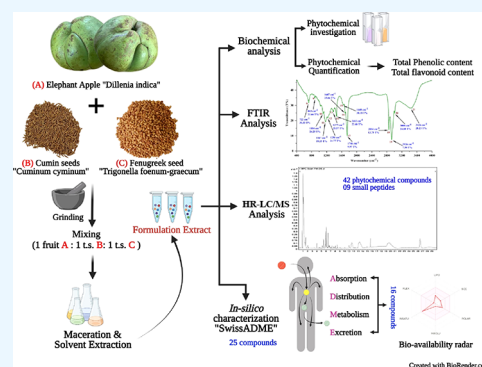
INTRODUCTION

Diabetes, characterized by abnormal metabolism of carbohydrates, lipids, and lipoproteins, has become one of the most common disorders, emerging as a worldwide epidemic.[1−3] According to a WHO 2014 report, the prevalence of diabetes in adults increased from 108 million reported in 1980 to over 442 million reported in 2014, and as of 2014 estimates, prevalence has increased significantly among population living in developing and underdeveloped nations.[4] Although there are numerous modern medications such as insulin injection or oral drugs such as hypoglycemic pills for diabetes management available in the pharmaceutical markets, most of them are associated with several concerns such as high cost or side effects including weight gain, hypoglycemia, gastrointestinal (GI) disturbances, and liver toxicity.[5] Medicinal herbs have remained popular as an alternative medicine due to their low cost, effectiveness, and historical, cultural, and religious preferences.[6] According to the World Health Organization,

due to poverty and a lack of access to modern medicine, 65−80% of the world's population in developing countries rely mostly on natural products for their primary health care.[7] The global market for medicinal plants increased from US $23 billion in 2002[8] to over US $83 billion in 2008.[9] Even though drug discovery from medicinal plants and herbs has been carried out extensively since World War I, somewhere around 10% of the world's 2.5 million species have been investigated for therapeutic potential, leaving much more waiting for discovery.[10,11]

Table 1. Preliminary Investigation of Phytochemical Constituents in Various Extracts of the Formulation[a]

		various extracts of the formulation				
phytochemical test		water	methanol	ethanol	ethyl acetate	chloroform
alkaloids	Mayer test	−	+	+	−	−
	Wagner test	+	+	+	+	+
tannins and phenolic compounds	FeCl₃ test	+	+	+	−	−
	gelatin test	−	+	+	+	+
	dil. iodine test	−	−	−	−	+
flavonoids	NaOH and acid test	+	+	+	+	+
	lead acetate test	+	+	+	−	+
fixed oil and fat	spot test	+	+	+	+	+

[a]+ indicates the presence and − indicates the absence of phytochemicals in their respective extract of the formulation.

Plants always have been a great source of drugs, and most of the drugs that are presently available have been originally derived from plants, either directly or indirectly.[3] India is recognized as the world's botanical garden because it produces most of the medicinal plants.[12] Among the list of 21,000 medicinal plants (complied by the WHO) used for therapeutic purposes around the world, 2500 different plant species have been reported in India and 150 species of them are being used economically on a large scale.[13] Among 450 verified medicinal plants with antidiabetic potential, only 109 plants are known for their mechanism of action.[14] The discovery of novel medicinal plants extensively relies on traditional knowledge and the historical literature.[15] Because of the efficacy in human clinical trials and the low side effects of drugs derived from medicinal herbs, medicinal plants have emerged as a major priority in the search of novel anti-diabetic medicine.[16,17] Because of the increasing prevalence, there is a growing need to establish an integrated approach for diabetes management and prevention by exploring the efficacy of traditional herbal remedies.[18] There are several reports highlighting a variety of medicinal plants and plant-based formulations that exhibit antidiabetic properties.

Dillenia species have been reported for their medicinal properties and utilized as complementary medicine for a long time (by various countries such as India, Indonesia, Thailand, Vietnam, Malaysia, *etc.*) either alone or in the form of formulations. In India, different plant parts of *Dillenia* species such as *pentagyna* (bark, leaf, and fruit), *indica* (fruit, leaf, flower, and bark), *andamanica* (stem bark) have been utilized as alternative medicine for the treatment of diabetes,[3,19−21] cancer,[20,22] wounds and burns,[22,23] constipation,[23] pain relief,[20,22,24,25] jaundice,[26] fever and fatigue,[27,28] dysenteries,[20,22,29] and diarrhea and to increase appetite.[27] Apart from that, other herbs such as fenugreek (*Trigonella foenum-graecum*), a medicinal plant of the Leguminosae family,[30] and cumin (*Cuminum cyminum*), an aromatic plant of the Apiaceae family,[31−33] are other well-known species and have been utilized for a very long time as food and natural medicine,[34] and their great nutritional value has made them more attractive to the researchers. Both plants and plant parts have been used in folklore therapy and traditional medicines and are reported for their anti-microbial, anti-diabetic, anti-inflammatory, and antioxidant activities.[31,35−38] The objective of this study is to perform a preliminary investigation of potential phytochemicals; to quantify the total phenolic and flavonoid contents; and to identify and characterize potential phytochemicals using Fourier transform infrared (FT-IR) spectroscopy analysis, high-resolution liquid chromatography mass spectrometry (HR-LC−MS) analysis, and *in silico* characterization of

physiochemical, drug-likeness, and pharmacokinetic properties of identified phytochemical compounds from various extracts of the formulation, which had been utilized by traditional healers from Northeast India for the management of diabetes mellitus.

■ RESULTS AND DISCUSSION

Preliminary Phytochemical Screening. Medicinal plants and herbs are enriched with essential phytochemicals, which are a variety of primary and secondary plant metabolites responsible for anti-hyperglycemic, anti-inflammatory, anti-diabetic, and anti-microbial effects and other known biological activities.[6,12] Five freshly prepared extracts were subjected to the preliminary phytochemical screening (see the Materials and Methods section) to identify the presence of alkaloids, tannins, phenolics, flavonoids, fixed oil, and fats in the formulation. The phytochemical profile from preliminary investigation revealed that the various extracts of the formulation are enriched with a variety of essential phytochemicals including alkaloids, phenolics, tannins, flavonoid compounds, fixed oil, and fats, as depicted in Table 1. Methanolic and ethanolic extracts of the formulation show similar phytoconstituent profiles and reveal a negative result in the dilute iodine test for tannins and phenolic compounds, while the acetate extract resulted in four negative results in the Mayer test, FeCl₃ test, dilute iodine test, and lead acetate test for alkaloids, phenolics, and flavonoids, respectively. All extracts confirm the presence of fixed oil and fats in the formulation. Alkaloid, phenolics and tannins, flavonoids, fixed oil, and fats are the most essential type of phytochemicals and have also been independently reported in several other investigations of various species of *Dillenia* and in fenugreeks and cumin herbs.[31,32,36] Analyzed phytoconstituents in various extracts of the formulation have been reported to possess medicinal importance in various pathological conditions such as diabetes, cancer, and inflammation.

Determination of the Total Phenolic and Flavonoid Content in Various Extracts of the Formulation. The phenolic and flavonoid compounds are naturally occurring compounds with substantial antioxidant capacity that can be found in various parts of plants. Phenolic and flavonoid compounds can accept an electron from reactive oxygen species, resulting in phenoxyl radicals that are considerably more stable. The presence of phenolics and flavonoids can interrupt the chain reaction of reactive oxygen species in cellular mechanisms and protect the human body from reactive oxygen species-induced damages. Because of the cardio-protective, anti-cancer, anti-diabetic, anti-aging, and neuro-

protective nature of phenolic and flavonoid compounds, plants enriched with them may enhance our body's antioxidant capacity.[39] As a result, determining these compounds in plants is essential. The total phenolic and flavonoid content determined in various extracts of the formulation are given in Table 2. The phenolic content was expressed in terms of

Table 2. Estimated Concentration of the Total Phenolic and Flavonoid Contents Present in Respective Extract of the Formulation

| extract	TPC (mg GAE/g)	TFC (mg QE/g)
water	14.89 ± 0.46	13.71 ± 0.53
methanol	33.68 ± 0.22	49.96 ± 1.99
ethanol	35.61 ± 0.15	45.41 ± 0.67
ethyl acetate	22.62 ± 0.47	64.13 ± 2
chloroform	19.15 ± 0.31	76.33 ± 2.96

gallic acid equivalents (GAE) using the standard curve equation ($y = 0.0073x - 0.0633$) with a correlation coefficient (R^2) of 0.9799 [Figure 1a]. The highest content of total phenolic compounds was quantified in the ethanolic extract with a value of 35.61 ± 0.15 mg GAE/g, followed by the methanolic extract with a content of 33.68 ± 0.22 mg GAE/g, while the hot water extract showed the least content 14.89 ± 0.46 mg GAE/g, as shown in Figure 1b and Table 2. In selected organic solvents, the decreasing order of phenolic

content is as follows: ethanolic extract > methanolic extract > ethyl acetate extract > chloroform extract > water extract.

Previous studies on different parts of *Dillenia pentagyna* revealed that the highest total phenolic content was present in the bark (9.66 ± 0.06) and leaves (9.15 ± 0.03), while fruit and seed extracts showed the lowest content 0.86 ± 0.02 and 0.75 ± 0.03 mg GAE/g, respectively.[40] Akbari *et al.* reported a 38.97 ± 0.34 mg GAE/g phenolic content in fenugreek seed oil.[38] Previously, Mohamed *et al.* determined (23.02 ± 0.045) mg GAE/g of phenolic content in the ethanol extract of *C. cyminum* L. seeds.[41]

The total flavonoid content of the extract was expressed in terms of quercetin equivalents (QE) using the standard curve equation in ($y = 0.004x - 0.0218$) with a correlation coefficient (R^2) of 0.9903 [Figure 1c]. Here, the highest content of flavonoid was quantified in the chloroform extract, 76.33 ± 2.96 mg QE/g, while the water extract showed the least content of flavonoids, 13.71 ± 0.53 mg QE/g, as shown in Figure 1d. The decreasing order of the flavonoid content among all selected organic solvents is as follows: chloroform extract > ethyl acetate extract > methanolic extract > ethanolic extract > water extract.

A previous report on various parts of *D. pentagyna* revealed the highest flavonoid content to be in the bark extract (42.12 ± 2.42) milligram rutin equivalents per gram dry weight of the extract, while sepals was determined to have the least content (6.08 ± 0.35) milligram rutin equivalents per gram dry weight of the extract.[40] Previously, Akbari *et al.* reported 14.417 ±
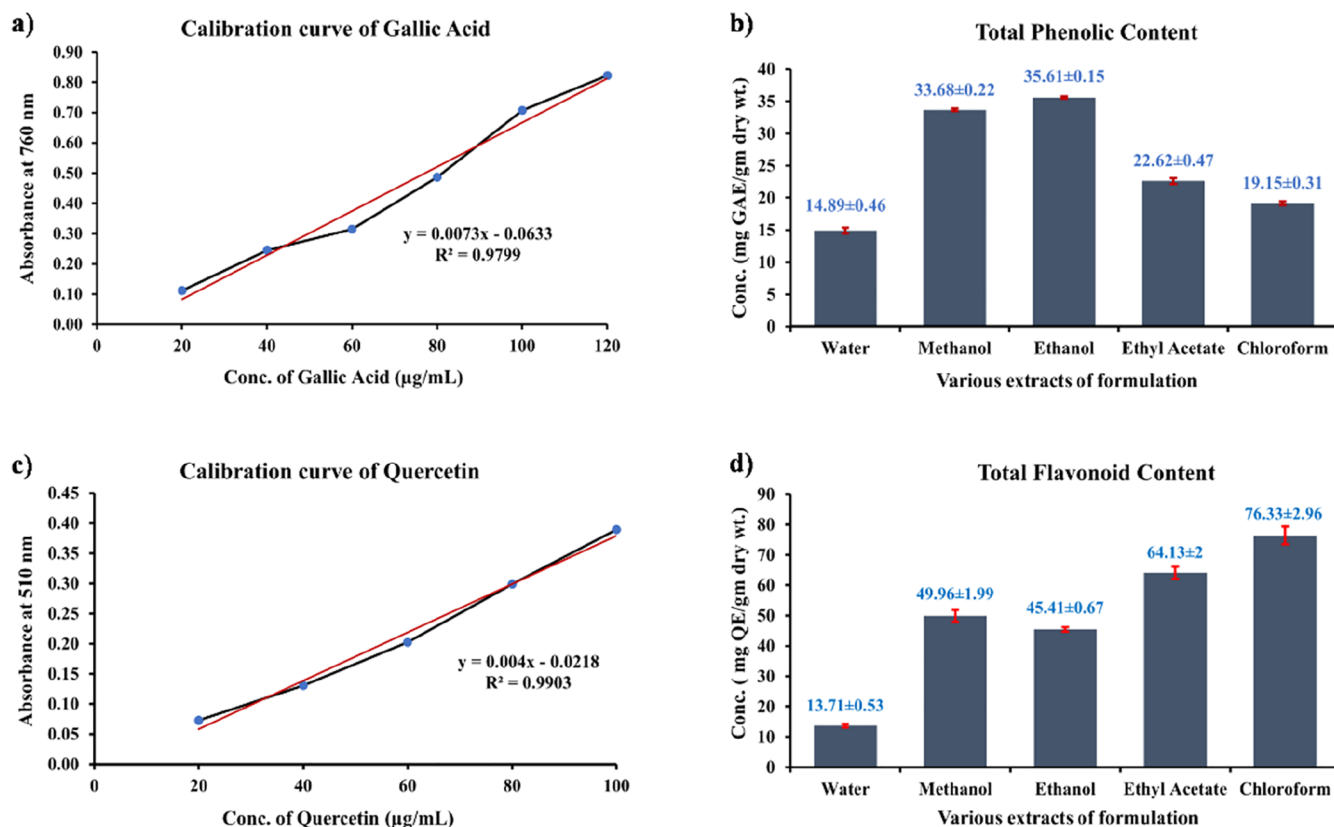
Figure 1. Estimation of phytochemical content in various extracts of the formulation: (a) calibration curve for the quantification of the total phenolic content in various extracts of the formulation using gallic acid as a standard compound, (b) concentration of the total phenolic content (mg GAE/g sample) present in respective extract of the formulation; (c) calibration curve for the estimation of the total flavonoid content in various extracts of the formulation using quercetin as a standard compound, and (d) concentration of the total flavonoid content (mg QE/g sample) present in respective extract of the formulation.
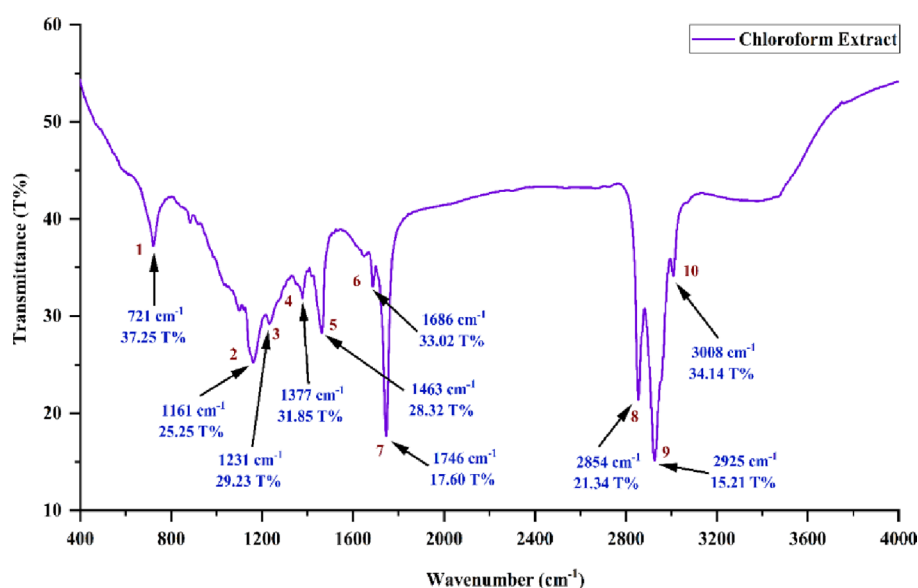
Figure 2. FT-IR spectrum representing potential bands in the chloroform extract of the formulation.

Table 3. All Potential Bands, Corresponding Functional Groups, and Possible Compounds Identified in the Chloroform Extract of the Formulation Using FT-IR Spectroscopy

| wavenumber (cm^{-1})					
band range (literature) (cm^{-1})	band range (experimental)	band no.	band interaction	band assignments	possible compounds
1000−650	721	1	bend	C=C	alkene
1400−1000	1161, 1231	2, 3	stretch	C−N/F	amine and fluoro compound
	1161	2	stretch	C−O	tertiary alcohol
	1231	3	stretch	C−O	alkyl aryl ether
	1377	4	bend and stretch	O−H and C−F	phenol, alcohol, and fluoro compound
1600−1300	1463	5	bend	C−H	alkane
2000−1650	1686	6	stretch	C=O	conjugated acid and conjugated aldehyde
	1746	7	stretch	C=O	ester, δ-lactone, and cyclopentanone
4000−2500	2854, 2925	8, 9	stretch	C−H, N−H, and O−H	alkane, amine salt and alcohol, and carboxylic acid
	3008	10	stretch	C−H and O−H	alkene and alcohol, carboxylic acid

0.23 mg QE/g flavonoids in fenugreek seed oil.[38] The flavonoid content in the ethanolic extract of *C. cyminum* L. seeds was observed to be 19 ± 0.132 mg QE/g.[41]

FT-IR Analysis of Various Extracts of the Formulation. Flavonoids, a class of hydroxylated phenolic compounds, possess antioxidant capacity and have gained a lot of attention as potential treatments for diseases caused by free radicals, including diabetes mellitus.[42−44] In the quantitative analysis of the total flavonoid contents in various extracts of the formulation, we find that the chloroform extract contains the highest content, followed by the ethyl acetate extract, and the least content is contained in the water extract, as shown in Table 2. We further demonstrate the functional group of active components present in the chloroform extract (Figure 2), water extract, and ethyl acetate extract (Figure S1) based on band values identified in the FT-IR spectrum. All potential bands were labeled in numeric values (Figures 2 and S1), and possible functional group band values are represented in Tables 3 and S1. The flavonoid-enriched chloroform extract of the formulation showed 10 significant bands (between 400 and 4000 cm^{-1}), and most of these bands share their corresponding spectral range with the water extract and ethyl acetate

extract (see Figure S1 and Table S1) of the formulation, as mentioned below.

In the chloroform extract of the formulation, band (1) at 721 cm^{-1} identified in the wavenumber range of 1000−650 cm^{-1} indicates C=C bending of alkenes, also shared with the ethyl acetate extract bands (18) and (19) (Figure S1 and Table S1). All three bands identified in the wavenumber range of 1400−1000 cm^{-1}, including band (2) at 1161 and (3) at 1231 cm^{-1}, representing C−N stretching of the amine and C−F stretching of fluoro compounds, are also shared with two bands (12 and 13) of the water extract and three bands (20−22) of the ethyl acetate extract. Band (2) also indicates C−O stretching of the tertiary alcohol, and band (3) is attributed to alkyl aryl ether stretching. However, band (4) at 1377 cm^{-1} showed O−H bending of alcohols and phenols and C−F stretching of fluoro compounds. One band identified between 1600 and 1300 cm^{-1}, band (5) at 1463 cm^{-1}, indicates C−H bending of alkanes, also shared with band (24) of the ethyl acetate extract. In the range of 2000−1650 cm^{-1}, two potential bands (6 and 7) are observed at 1686 and 1746 cm^{-1}, representing C=O stretching of conjugated acids and aldehydes and C=O stretching of ester, δ-lactone, and cyclopentanone, respectively, which are shared with bands
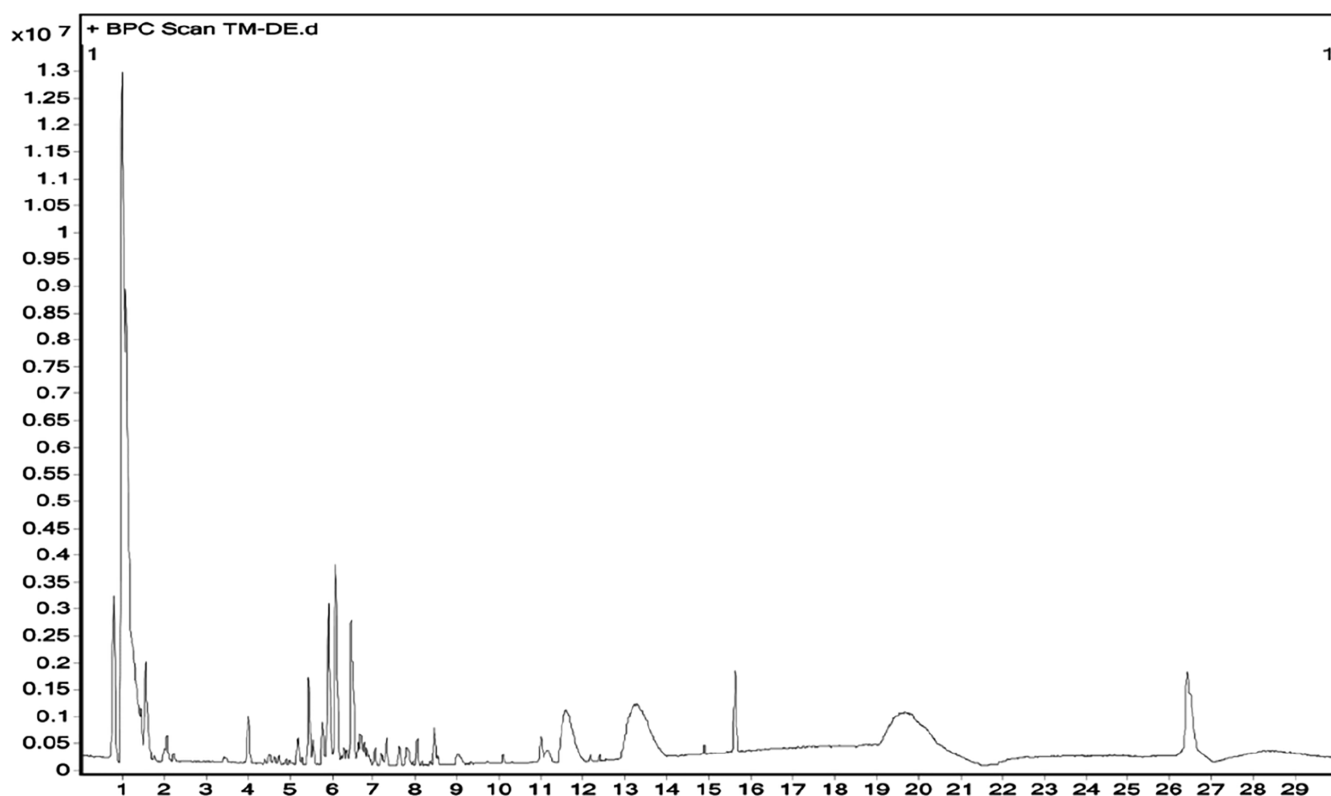
Figure 3. Chromatograms of identified phytochemical constituents' profile in the methanolic extract of the formulation using the HR-LC/MS technique.

(26) and (27) of the ethyl acetate extract. Among the remaining three bands identified in the range of 4000−2500 cm^{-1}, two bands (8 and 9) at 2854 and 2925 cm^{-1} wavenumbers represent C−H, N−H, and O−H stretching of alkane, amine salt and alcohol, and carboxylic acid compounds, also shared with band (16) of the water extract and bands (28 and 29) of the ethyl acetate extract. However, band (10) at 3008 cm^{-1} represents C−H and O−H stretching of alkene, alcohol, and carboxylic acid, also shared with band (30) of the ethyl acetate extract. Among all identified significant bands in the chloroform, water, and ethyl acetate extracts of the formulation, 11 bands showed O−H stretching of alcohols (Tables 1 and S1). Among these 11 bands, four bands (4, 8, 9, and 10) were identified in the chloroform extract, while two bands (16 and 17) were observed in the water extract and five bands (13, 18, 19, 20, and 21) were observed in the ethyl acetate extract, and most of them shared a common spectral range. The OH group plays a crucial role in anti-diabetic, antioxidant, and anti-bacterial activities.[45]

Phytochemical Composition: HR-LC−MS Analysis and Bioinformatics' Findings. The methanolic extract of the formulation was subjected to HR-LC/MS for the identification of phytoconstituents based on their retention time, experimental *m/z*, MS/MS fragments, database differences (library), metabolite class, and proposed compounds. MS data were obtained in the positive ionization mode. Most of the *m/z* values in our methanolic extract of the formulation were in the range between 130 and 465. Figure 3 depicts the HR-LC−MS chromatogram obtained for the methanolic extract of the formulation. Table 4 represents the 42 identified potential phytochemical compounds from the HR-LC/MS analysis of the methanolic extract of the formulation. The

ADME parameters of the identified compounds were estimated using an online server, SwissADME, to identify the most promising compounds with a limited risk of drug attrition in the later phase. Only analogues with the most significant ADME features have been considered in this study. Based on Lipinski's rule, the selected compounds, represented in Table 5, exhibit good oral absorption with good bioavailability scores (0.55−0.56) and TPSA (topological polar surface area) values less than 140 Å^2, suggesting that they will be anticipated to be orally absorbed. In the context of pharmacokinetics, all the selected compounds had high GI absorption characteristics (except 26) and also an ability to cross the blood−brain barrier (BBB) (except 2−4, 6, 7, 11, 19, 21, 26, 30, and 31). Some of them were expected to be non-P-gp substrates, which means that they have promising intestinal absorption and bioavailability. According to the screening data of CYP (cytochrome P450) enzyme isoforms (used in biotransformation of medicines and xenobiotics), most of the selected compounds were determined to be inhibitors of isoenzymes among the examined CYP1A2, CYP2C19, CYP2D6, and CYP3A4 isoforms. Because of the negative skin permeability values, they are not permeable through the skin and thus are not suitable candidates for transdermal drug delivery. Further, drug-likeness properties of selected compounds were examined using the bioavailability radar to anticipate their oral bioavailability. The result showed that at least 16 compounds, out of the 25 potential compounds observed for ADME parameters among the identified library of 42 compounds, fall in the pink area of the polygon (Figure 4), indicating their good oral bioavailability.

Moreover, HR-LC/MS analysis also reveals a total of nine small peptides (three di-peptides and six tripeptides)-like

Table 4. Phytochemical Compounds Identified in the Methanolic Extract of the Formulation Using the HR-LC−MS Technique[a]

entry (no.)	compounds	RT	MW	formula	[m/z]	DB diff (ppm)	hits (DB)
1	betaxolol	0.796	307.2099	$C_{18}H_{29}NO_3$	308.2173	15.7	3
2	norcotinine	0.815	162.0746	$C_9H_{10}N_2O$	185.0647	29.08	1
3	sulfabenzamide	0.817	276.056	$C_{13}H_{12}N_2O_3S$	299.0456	3.1	1
4	p-chlorobenzenesulfonylurea	0.819	233.9919	$C_7H_7ClN_2O_3S$	256.981	−22.7	1
5	10-keto tridecanoic acid	0.82	228.17	$C_{13}H_{24}O_3$	251.1592	11.15	8
6	carteolol	0.832	292.1737	$C_{16}H_{24}N_2O_3$	293.181	17.01	1
7	agmatine	0.838	130.1211	$C_5H_{14}N_4$	293.181	5.64	1
8	isoamyl nitrite	0.965	117.0779	$C_5H_{11}NO_2$	140.0671	9.32	8
9	3-pyridylacetic acid	1.017	137.0467	$C_7H_7NO_2$	138.054	7.19	9
10	flunixin	1.062	296.0731	$C_{14}H_{11}F_3N_2O_2$	319.063	14.05	1
11	vigabatrin	1.084	129.0783	$C_6H_{11}NO_2$	130.0856	5.18	4
12	phenylpropionylglycine	1.134	207.0886	$C_{11}H_{13}NO_3$	208.0958	4.54	6
13	tolnaftate	1.148	307.1046	$C_{19}H_{17}NOS$	330.0935	−4.79	8
14	neuraminic acid	1.222	267.0961	$C_9H_{17}NO_8$	268.1034	−2.4	8
15	diethylstilbestryl disulfate	1.246	428.0591	$C_{18}H_{20}O_8S_2$	451.048	1.93	1
16	metoclopramide	1.273	299.1364	$C_{14}H_{22}ClN_3O_2$	300.1435	12.29	1
17	2-amino-4-oxo-6-(1,2-dioxoprolyl)-7,8-dihydr-oxypteridine	1.296	267.0588	$C_9H_9N_5O_5$	268.066	5.73	1
18	anabasamine	1.452	253.1539	$C_{16}H_{19}N_3$	276.1432	15.69	1
19	indospicine	1.547	173.118	$C_7H_{15}N_3O_2$	196.1072	−9.22	3
20	5-(2-hydr-oxyethyl)-4-methyl thiazole	1.574	143.0397	C_6H_9NOS	144.0469	5.25	2
21	methyl o-methoxy-hippuric acid	2.068	223.0838	$C_{11}H_{13}NO_4$	224.0911	2.88	3
22	2,6-nonadienal	5.211	138.1037	$C_9H_{14}O$	139.111	5.56	5
23	glutaconic acid	5.461	130.028	$C_5H_6O_4$	153.0172	−10.74	6
24	eudesmic acid	5.461	212.0678	$C_{10}H_{12}O_5$	213.0751	3.2	6
25	2E,4E,6E,8E-decatetraenedioic acid	5.93	194.0572	$C_{10}H_{10}O_4$	195.0642	3.53	9
26	gentiopicrin	5.932	356.1098	$C_{16}H_{20}O_9$	379.0989	2.69	2
27	quercitrin	5.962	448.1	$C_{21}H_{20}O_{11}$	449.1073	1.28	1
28	homoveratric acid	6.359	196.0729	$C_{10}H_{12}O_4$	197.0801	3.44	10
29	clotrimazole	6.47	344.1101	$C_{22}H_{17}ClN_2$	367.0991	−5.98	2
30	hydralazine	6.471	160.0751	$C_8H_8N_4$	183.0642	−1.13	10
31	metanephrine	6.492	197.1068	$C_{10}H_{15}NO_3$	220.096	−8.36	7
32	cosmosiin	6.509	432.1048	$C_{21}H_{20}O_{10}$	433.112	2.04	3
33	tuberonic acid	6.715	226.1222	$C_{12}H_{18}O_4$	249.1115	−7.66	4
34	9-oxo-2E-decenoic acid	6.848	184.1116	$C_{10}H_{16}O_3$	207.1008	−9.02	5
35	demeclocycline	7.617	464.0948	$C_{21}H_{21}ClN_2O_8$	465.1019	8.25	1
36	ethyl everninate	8.039	210.0885	$C_{11}H_{14}O_4$	211.0957	3.59	7
37	valeryl salicylate	8.448	222.0885	$C_{12}H_{14}O_4$	223.0958	3.19	4
38	C16 sphinganine	11.606	273.2661	$C_{16}H_{35}NO_2$	274.2733	2.58	1
39	2-amino-tetradecanoic acid	13.666	243.2189	$C_{14}H_{29}NO_2$	244.2261	4.01	1
40	2R-amino hexadecanoic acid	15.629	271.25	$C_{16}H_{33}NO_2$	272.2572	4.03	4
41	levmetamfetamine	26.414	149.1193	$C_{10}H_{15}N$	150.1267	7.46	5
42	dextroamphetamine	26.446	135.1035	$C_9H_{13}N$	136.1107	9.87	2

[a]RT: retention time, MW: molecular weight, and [m/z]: mass divided by charge numbers.

proteins of molecular weight in the range of 228 to 405 g/mol, identified by comparison of spectrum data of the methanolic extract of the formulation with those of known compounds, as mentioned in Table 6. Identified peptides were composed of a majority of essential and non-essential amino acids distributed unevenly. Glycine, proline, glutamine, arginine methionine, and lysine were the most abundant amino acids, accounting for 70.83% of the total amino acids, while glutamic acid, asparagine, isoleucine, histidine, alanine, cystine, and valine were the least abundant amino acids, accounting for 29.166% of the total amino acids. Essential amino acids (methionine, isoleucine, lysine, histidine, and valine) together accounting for 29.168% of the total amino acids were found in six small peptides out of nine peptides. Positively charged amino acids (arginine, lysine, and histidine) together accounting for 25% of

the total amino acids were found in six small peptides out of nine peptides, while the negatively charged amino acid glutamic acid was the least abundant, with a content of 4.166% of the total amino acids, and was identified in only one peptide out of nine peptides. There was no aromatic amino acid identified in the methanolic extract of the formulation. Sulfur-containing side amino acid was found in three small peptides out of nine, which accounted for 12.5% of the total amino acids. The repertoire of peptides of the methanolic extract appeared to be more hydrophobic than hydrophilic from the amino acid composition, with hydrophobic amino acids accounting for 50% of the total amino acids present in eight peptides out of nine small peptides (Table 6).

Table 5. In Silico Analysis of Physiochemical and Pharmacokinetic Properties of Selected Phytochemical Compounds, Identified from the HR-LC/MS Analysis of the Methanolic Extract of the Formulation[a]

entry no.	MW (g/mol)	no. of heavy atoms	no. of arom. heavy atoms	frac Csp3	no. rotatable bonds	no. of H-bond accept	no. of H-bond donors	molar refract	TPSA (Å²)	consen Log $P_{o/w}$	Lipinski's rule	bioavailability score	GI absorp	BBB perm	P-gp substr	inhibitor CYP-1A2	CYP-2C9	CYP-2D6	CYP-3A4	Log K_p (skin perme) (cm/s)
1	307.43	22	6	0.67	11	4	2	88.9	50.72	2.9	yes	0.55	high	yes	no	no	no	yes	no	-6.18
2	162.19	12	6	0.33	1	2	1	48.43	41.99	0.65	yes	0.55	high	no	no	no	no	no	no	-7.50
3	276.31	19	12	0	4	3	2	71.65	97.64	1.35	yes	0.55	high	no	no	no	no	no	no	-7.49
4	234.66	14	6	0	3	3	2	50.64	97.64	0.76	yes	0.55	high	no	yes	no	no	no	no	-7.09
6	292.37	21	6	0.56	6	4	3	85.88	70.59	1.64	yes	0.55	high	no	no	no	no	yes	no	-7.39
7	130.19	9	0	0.8	4	2	3	37.96	90.42	-0.66	yes	0.55	high	no	no	no	no	no	no	-8.19
8	117.15	8	0	1	4	3	0	31.97	38.66	1.05	yes	0.55	high	yes	no	no	no	no	no	-5.79
11	129.16	9	0	0.5	4	3	2	34.96	63.32	-0.07	yes	0.55	high	no	no	no	no	no	no	-8.62
13	307.41	22	16	0.11	4	1	0	96.93	44.56	4.66	yes	0.55	high	yes	no	yes	no	no	no	-4.30
16	299.8	20	6	0.5	8	3	2	82.28	67.59	2.28	yes	0.55	high	yes	no	yes	no	yes	no	-6.27
18	253.34	19	12	0.38	2	3	0	81.17	29.02	2.33	yes	0.55	high	yes	yes	no	no	yes	yes	-6.74
19	173.21	12	0	0.71	6	4	4	46.54	113.19	-1.13	yes	0.55	high	no	no	no	no	no	no	-9.61
20	143.21	9	5	0.5	2	2	1	38.01	61.36	1.2	yes	0.55	high	yes	no	no	no	no	no	-6.57
21	223.23	16	6	0.27	6	4	1	56.83	64.63	1.15	yes	0.55	high	no	no	no	no	no	no	-7.01
22	138.21	10	0	0.44	5	1	0	44.63	17.07	2.34	yes	0.55	high	yes	no	no	no	no	no	-5.57
26	356.32	25	0	0.56	4	9	4	80.45	134.91	-0.8	yes	0.56	low	no	yes	no	no	no	no	-9.35
29	344.84	25	23	0.05	4	1	0	101.84	17.82	4.64	yes[#]	0.55	high	yes	yes	yes	yes	yes	yes	-4.56
30	160.18	12	10	0	1	3	2	46.74	63.83	0.82	yes	0.55	high	no	no	yes	no	no	no	-6.57
31	197.23	14	6	0.4	4	4	3	53.5	61.72	0.59	yes	0.55	high	no	no	no	no	no	no	-7.96
36	210.23	15	6	0.36	4	4	1	56.01	55.76	2.15	yes	0.55	high	yes	no	yes	no	no	no	-5.69
38	273.45	19	0	1	14	3	3	84.06	66.48	3.7	yes	0.55	high	yes	yes	no	no	yes	no	-4.62
39	243.39	17	0	0.93	12	3	2	73.89	63.32	2.71	yes	0.55	high	yes	yes	no	no	no	no	-5.80
40	271.44	19	0	0.94	14	3	2	83.51	63.32	3.42	yes	0.55	high	yes	yes	no	no	yes	no	-5.20
41	149.23	11	6	0.4	3	1	1	48.63	12.03	2.25	yes	0.55	high	yes	no	no	no	yes	no	-5.74
42	135.21	10	6	0.33	2	1	1	43.73	26.02	1.94	yes	0.55	high	yes	no	yes	no	no	no	-5.88

[a]Table abbreviations: frac, fraction; accept, acceptors; refract, refractivity; consen, consensus; Log Po/w, partition coefficient between n-octanol and water; GI absorp, gastro-intestinal absorption; BBB perm, blood−brain barrier permeant; P-gp substr, permeability of glycoprotein substrate; CYP, cytochrome P450; perme, permeation. [#]1 violation: MLOGP > 4.15. MLOGP > 4.15.
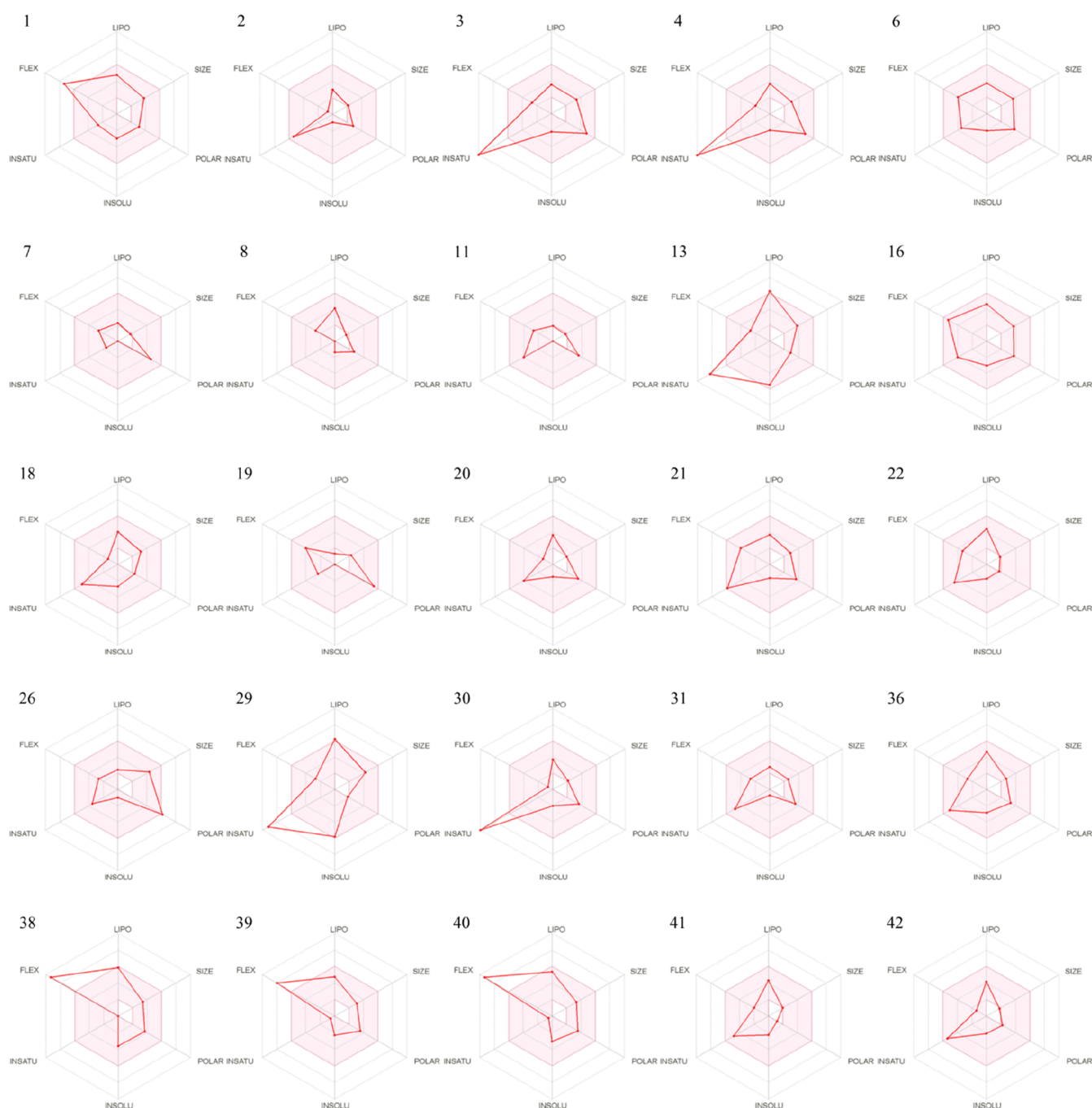
Figure 4. Bioavailability radar of selected phytochemical compounds identified in the methanolic extract of the formulation using the HR-LC−MS technique, based on physicochemical indices ideal for oral bioavailability. The pink zone in the bioavailability radar is the ideal physicochemical space for oral bioavailability. LIPO (lipophilicity: $-0.7 < XLOGP3 < p\ 5$); SIZE (molecular weight: 150 g/mol < mol wt < 500 g/mol); POLAR (polarity: 20 Å^2 < TPSA < 140 Å^2); INSOLU [insolubility: $0 < \log S$ (ESOL) < 6]; INSATU (insaturation: 0.25 < fraction C sp^3 < 1); and FLEX (flexibility: 0 < number of rotatable bonds < 9).

■ CONCLUSIONS

The ethnobotanical plants are enriched with bio-active phytochemical compounds having promising pharmacological activities and can be utilizes as a therapeutic alternative. Many researchers and scientists had been seeking to discover new pharmacologically active phytomolecules from the plants, specifically from herbs, to target the various pathophysiological diseases such as cancer, cardiovascular disease, and metabolic disorders for a long time. In this study, we attempted to scientifically validate the polyherbal formulation, traditionally used by tribal communities of the northeastern part of India for the treatment of diabetes. Plant parts and herb seeds utilized to prepare the formulation have also previously been studied independently and reported for their medicinal properties against various diseases including anti-diabatic, anti-cancer, anti-inflammatory and many other properties. To the best of our knowledge, the combination of this polyherbal formulation and its characterization by preliminary phytochemical screening along with the quantification of the total phenolic and flavonoid content in the various extracts of the formulation has

Table 6. HR-LC/MS Analysis Revealed Peptide-Like Proteins Identified in the Methanolic Extract of the Formulation

entry (no.)	small peptides	retention time	molecular weight	formula	[m/z]
43	Ile Pro	1.194	228.1467	$C_{11}H_{20}N_2O_3$	229.154
44	Arg Pro	1.353	271.1647	$C_{11}H_{21}N_5O_3$	294.1539
45	Met Arg	2.05	305.1495	$C_{11}H_{23}N_5O_3S$	328.1385
46	Lys Gly Gly	1.578	260.1502	$C_{10}H_{20}N_4O_4$	283.1395
47	Gly His Pro	1.101	309.1414	$C_{13}H_{19}N_5O_4$	310.1485
48	Ala Cys Val	1.14	291.1273	$C_{11}H_{21}N_3O_4S$	314.1165
49	Arg Asn Gly	5.559	345.1783	$C_{12}H_{23}N_7O_5$	346.1855
50	Gln Met Lys	6.771	405.1995	$C_{16}H_{31}N_5O_5S$	406.2066
51	Glu Gln Gln	1.27	403.171	$C_{15}H_{25}N_5O_8$	426.1602

not been performed yet. HR-LC—MS-based identification of potential phytochemicals has also been reported for the first time, and the profile reveals 42 compounds, out of which 25 compounds showed significant physiochemical and pharmacokinetic profiles, and of these 25, 16 compounds showed promising bioavailability, as predicted by the *in silico* analysis of the identified phytochemical compounds. FT-IR analysis of flavonoid-enriched extracts of the formulation also confirmed the presence of −OH functional groups among phytochemical compounds, indicating the possibility of an antioxidant nature. Quantitative analysis confirmed that the formulation is enriched with phenolic and flavonoid contents, and previous studies conducted on the various species, parts of plants, and herbs utilized to prepare the formulation have also been reported.

■ MATERIALS AND METHODS

Reagents and Solution Preparations. All chemicals used in this study were analytical grade reagents. Methanol, ethanol, chloroform, ethyl acetate, bromocresol green, Folin—Ciocalteu reagent, sulfuric acid (H_2SO_4), and hydrochloric acid (HCl) were purchased from Merck (Pvt. Ltd. Mumbai, India). Sodium phosphate buffer, mercuric chloride, gelatin, sodium chloride, ferric chloride, lead acetate, sodium carbonate, citric acid, and sodium hydroxide were obtained from HiMedia (Mumbai, India). Aluminum chloride, quercetin, and gallic acid were purchased from Sigma (Bangalore, India). Sodium nitrite, potassium iodide, iodine, and sodium chloride were obtained from SRL Pvt. Ltd. Mumbai, India.

Instrumentation. An FT-IR spectroscope (Spectrum-100, PerkinElmer, Singapore), an HR-LC—MS system (Agilent Technology, USA), a mortar and pestle (Sigma), tabletop centrifuge (Tarson), distilled water (Millipore), a vortex (Tarson Spinot), a spectrophotometer (Multiskan GO, Thermo Scientific), an electronic balance (Kern and Aicoset), a freezer (Samsung), and a pH meter (Eutech Instruments Pte Ltd) were used.

Collection of Samples and Extraction. Initially, elephant apple (*Dillenia indica* Linn.) was purchased from the local market of Tezpur, Assam on 24 February 2018. While two other samples including cumin seeds and fenugreek were purchased from the Patanjali store of the Tezpur market. Using

a sharp knife, sample A (elephant apple fruit, petals) was cut and subjected to preliminary processing, which includes sun-drying for 2 to 3 days. Sample A (sun-dried petals of elephant apple), sample B (cumin seeds), and sample C (fenugreek) were ground separately using a mortal and pestle and mixed in a ratio of 1 fruit (sample A)/1 teaspoon (sample B)/1 teaspoon (sample C) to prepare the final formulation. This formulation was further extracted in five different solvents including methanol, ethanol, ethyl acetate, chloroform, and hot water by dissolving 50 g of the prepared formulation in 200 mL of each solvent and subjected for 7 days of dark incubation at room temperature with occasional shaking. After 7 days of dark incubation, each solution mixture was filtered by using filter paper (Whatman No. 1 filter paper), followed by evaporation, and dried to a powdered form, which was utilized for further investigations.

Preliminary Phytochemical Screening. The preliminary phytochemical screening was performed with all five extracts of the formulation using chromophore reagents. For this, various extracts were taken in a 5 mL glass test tube for the preliminary investigation, following the procedure previously reported for qualitative identification and confirmation of phytochemicals presented in the samples.[46−52]

Test for Alkaloids. Each extract of the formulation was separately mixed with 5 mL of hydrochloric acid and filtered. Each filtrate was tested with the following reagents:

Mayer's Test. A few drops of Mayer's reagent were added to 2 mL of the filtrate in a test tube. Formation of green color or a white precipitate indicates the presence of alkaloids.

Wagner Test. A few drops of Wagner's reagent (a solution of iodine in potassium iodide) were mixed with 2 mL of the filtrate in a test tube. Formation of a reddish-brown precipitate indicates the presence of alkaloids.

Test for Tannins and Phenolic Compounds. Ferric Chloride Test. The extract was treated with a few drops of ferric chloride solution, and formation of a bluish black color indicates the presence of phenols and tannins.

Gelatin Test. The extract of the formulation was treated with 1% gelatin solution containing 10% sodium chloride. Formation of a precipitate indicates the presence of tannins and phenolic compounds.

Iodine Test. Small quantities of different extracts were treated with diluted iodine solution separately. Appearance of a transient red color indicates the presence of tannins and phenolic compounds.

Test for Flavonoids. NaOH and Acid Test. 1 mL of each extract of the formulation was separately treated with a few drops of dilute sodium hydroxide. Formation of an intense yellow color that becomes colorless upon mixing with a few drops of dilute HCl indicates the presence of flavonoids.

Lead Acetate Test. A small quantity of each extract was treated with lead acetate solution, and formation of a yellow color precipitate after a few minutes indicates the presence of flavonoids.

Test for Fixed Oil and Fats. Spot Test. A small quantity of different extracts was separately pressed between two filter papers. Appearance of an oil stain on the paper indicates the presence of fixed oil.

Estimation of the Total Phenolic Contents. The total phenolic contents present in various extracts of the formulation were determined using the Folin—Ciocalteu colorimetric method described by Singleton and Rossi[53] with a slight modification. Briefly, 0.50 mL of respective extracts in

methanol (1 mg/mL) was mixed with 0.75 mL of the Folin–Ciocalteu reagent after 5 min, and 0.4 mL of saturated sodium carbonate solution (about 75 g/L) was added into the reaction mixture. The final reaction mixture was incubated in the dark for 2 h at room temperature, and absorbance was measured at 760 nm using a UV–vis spectrophotometer. The total amount of phenolics in the respective extract of the formulation was calculated using a calibration curve obtained from the absorbance of gallic acid, as a standard, at different concentrations (20, 40, 60, 80, 100, and 120 μg/mL). The total phenolic content in the formulation was expressed as milligram gallic acid equivalent per gram dry weight of respective formulation extract (mg GAE/g extract of the formulation) and $\pm$SD (standard deviation) for three replicate analyses.

Estimation of the Total Flavonoid Contents. The total flavonoid content present in the various extracts of the formulation was determined using the aluminum chloride colorimetric method[54,55] with a slightly modification. Briefly, 400 μL of the formulation extracts in methanol (1 mg/mL) was mixed with 30 μL of 5% sodium nitrite. The reaction mixture was mixed well and incubated for 5 min at room temperature, and then, 30 μL of 10% AlCl$_3$·6H$_2$O solution was added, followed by the addition of 200 μL of 1 M NaOH after another 5 min of incubation at room temperature. The final volume of the reaction mixture was increased to 1 mL using distilled water. The final reaction mixture was incubated in the dark for 15 min at room temperature before measuring the absorbance at 510 nm using a UV–vis spectrophotometer. Similarly, the total amount of flavonoid content in the formulation was determined using a calibration curve prepared by measuring the absorbance of quercetin, as a standard, at different concentrations. The total flavonoid content was expressed as milligram quercetin equivalent per gram dry weight of the respective formulation extract (mg QE/g extract of the formulation) and $\pm$SD for three replicates.

FT-IR Spectroscopy Analysis. FT-IR spectroscopy was performed for various extracts of the formulation mixed with dried KBr salt using a mortar and pestle. The mixture of KBr salt with respective extract of the formulation will be compressed into the form of a thin salt disc. The salt disc was loaded onto an FT-IR spectroscope (Spectrum-100, PerkinElmer, Singapore) for further spectral measurement and scanned in the range of 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

HR-LC/MS Analysis of the Methanolic Extract. The methanolic extract of the formulation was used for HR-LC/MS analysis. The sample was sent to the Sophisticated Analytical Instrument Facility (SAIF) of Indian Institute of Technology, Bombay (IIT Bombay), India, for analysis. One-dimensional separation of phytochemicals from the methanol extract was carried out in the positive ion mode using a ChipCube, 6550 iFunnel Q-TOF B.05.01 (B5125) (Agilent Technologies, USA) mass spectrometer equipped with an electrospray ionization source. For chromatographic separation, a Hypersil GOLD C-18 (2.1 × 100 mm, particle size: 3 μm) column was used as the stationary phase and 3 μL of the injection volume was injected using a needle at an injection speed of 100 μL/min with a 5.0 sample flush out factor. For the mobile phase combination of "solvent A": 100% water (H$_2$O) (0.1% formic acid in water) and solvent B: 100% acetonitrile (CH$_3$CN) (90% acetonitrile, 0.1% formic acid and 10% water) were used at a flow rate of 300 μL/min. The gradient of the mobile phase started with 95:5 (H$_2$O/CH$_3$CN) for 20 min, then changed to 5:95 (H$_2$O/CH$_3$CN) for 5 min, and finally returned to 95:5 (H$_2$O/CH$_3$CN) for 5 min. The acquisition method was set to be MS—a minimum range of 125 (m/z) and a maximum of 1000 (m/z) with a scanning rate of 1 spectra/s. Settings for the iFunnel MS Q-TOF segment of instrument was maintained: a gas flow rate of 13 L/min at 250 °C temperature; a sheath gas flow rate of 11 L/min at 300 °C temperature, and 35 psi nebulizer gas flow pressure.

***In Silico* ADME Profile.** The physiochemical, lipophilicity, drug-likeness, and pharmacokinetic properties of phytochemical compounds, identified from the HR-LC–MS analysis of the methanolic extract of the formulation, were estimated using an online server for ADME (absorption, distribution, metabolism, and excretion) prediction, SwissADME (http://www.swissadme.ch/).

■ ASSOCIATED CONTENT

ⓈI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03117.

> FT-IR spectrum representing potential bands in the water and ethyl acetate extract of the formulation and all potential bands, corresponding functional groups, and possible compounds identified in the respective extract of the formulation using FT-IR spectroscopy (PDF)

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Author Contributions

P.K.S. conceived the project, T.M. supervised the experimental work, J.S. performed the *in silico* work, A.K. supervised the *in silico* work, and P.K.S., J.S., and A.K. performed data analysis and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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