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Development and *In-vitro* Evaluation of Antidiabetic Potential of Matrix Type Polyherbal Formulation

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ABSTRACT

Matrix type poly herbal formulation (PHF) was carried out by freeze drying method, by employing maltodextrin (MD), sodium alginate (SA) and gelatine (GE) with their designated different combinations as encapsulating wall materials. Various bioactive components like total phenol contents (TPC), total flavonoids content (TFC), antioxidant activity (i.e. DPPH and $ABTS^+$ [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid] assays), moisture contents, water activity (a_w), solubility, hygroscopicity, glass transition temperature (T_g), *in vitro* α-amylase and α-glycosidase inhibition and bioavailability ratios of the powders were investigated. Among all products, KD_1 (5% SA and 5% MD) and KD_4 (10% SA) have proven to be the best treatments with respect to the highest preservation of antioxidant components. These treatments also exhibited higher antioxidant potential by DPPH and an $ABTS^+$ assays. Moreover, the aforesaid treatments also demonstrated lower moisture content, water activity (a_w), hygroscopicity and glass transition temperature (T_g). All freeze dried samples showed irregular (asymmetrical) microcrystalline structures. KD_1 and KD_4 illustrated the highest *in vitro* anti-diabetic potential due to great potency for inhibiting α-amylase and α-glucosidase activities.

KEYWORDS

Matrix Poly herbal formulation, total phenolic content, total flavonoids content, *Salacia prenoids*, *Coccinia indica*, *Annona squamosa*

INTRODUCTION

A direct correlation between oxidative stress and insulin resistance (key factor for type-II DM) has been elaborated in mini review by *Hurrle et al*.[1] Amongst naturally-occurring antioxidants, polyphenols and their derivative compounds represent a diverse class of ubiquitous material, i.e., from simple molecules to complex configuration such as phenolic acids; hydroxybenzoic and hydroxycinnamic acids, hydrolysable and condensed tannins, and flavonoids, these are most important compounds for nutraceutical, therapeutics and pharmacological point of view.[2]

In order to counteract harmful effects of oxidative stress due to pathological conditions or physical exercise, human beings are often administered dietary supplements having supposed high antioxidant activities.[3] Oxygen free radical induced cellular damages have been implicated in many pathobiological conditions, viz. Malignancy, ageing process and degenerating diseases, etc.[4,5] The excess production of these reactive oxygen species (ROs) are considered serious issue for human health as their surplus generation can lead to different patho-physiological conditions like fast aging process via damaging the nucleic acids and changing in the conformation of proteins, heart-related disorders, diverse type of cancers, immunity related dysfunctions, inflammation, membranous lipid oxidation, decline of hydroperoxide synthesis, neurodegenerative disorders, lungs and kidney illness, UV-irradiation, osteoporosis/bone related diseases and health related diseases called 'oxidative stress'.[6] Naturally occurring antioxidants, polyphenols and their derivatives have revealed various health endorsing activities like anti-diabetic, anti-malarial, anticancer and antioxidant activities i.e. free radicals scavenging, declining of hydroperoxide development, hampering the lipid oxidation etc.[7] Some investigations illustrated that selected individual plants contained abundant quantity of polyphenols but their herbal combinations were found to produce best antioxidant activity among all individual extracts due to synergistic effect. Owing to synergism, polyherbal formulation demonstrated vast advantages over single herbal formulation.[8] Some advantages of polyherbal formulation (PHF) like superior restorative effect, low dosage requirement to acquire enviable pharmacological effect, consequently lessening the risk of harmful side effects. Additionally, PHF facilitates the patient's convenience by eradicating the need of taking more than one formulation at a time, which ultimately leads to better compliance and therapeutic effect.[9]

The degradation of natural antioxidants may hamper the possible effectiveness of these antioxidants in commercially available anti-diabetic drugs as polyphenols are incredibly sensitive to diverse range of circumstances, during food processing and storage practice likewise; high temperature of surrounding, incidence of oxygen and light, pH, existence of oxidative enzymes, moisture contents.[10-12]

Nowadays, plant/herb extracts are preferably matrix type using materials like maltodextrin (MD), sodium alginate (SA) and gelatin (GE). Maltodextrin is used preferably because of its low viscosity, high solubility in water and their solutions are monochromic in appearances. The use of sodium alginate polymer is one of the largest potential applications in the matirx herbal drugs because of their versatility, biocompatibility and toxicity exemption.[13] Sodium alginate has been used as the encapsulating material due to its ability to absorb water, to be easy manipulated and innocuousness, having also other features such as gelling, stabilizing and

thickening, reasons which have been of great interest to the herbal industry.[14] It is the most polysaccharide used as encapsulating material for herbal drugs and plant extracts, due to ease of handling, non-toxic nature and low cost, besides increasing the viability of these extracts when exposed to different conditions when are compared with non-encapsulated bacteria.[15] In addition, gelatine is also a better option for matrix formulations because of its superior characteristics for emulsification, film-formation, water solubility, last but not least ability to form finer dense complex. A sole encapsulating agent has been resolved by using different combination of polymers due to their effectiveness, eventually has been resolved by using different combination of polymers due to their diverse features. The selection of polymer's combinations which possibly consequence in superior encapsulating efficiency and regarded economically suitable than the single biopolymers has been becoming the point of emerging interest.[16]

Salacia prenoides also known as saptarangi in gujarati, is a tropical climbing shrub, with opposite simple leaves, and small inconspicuous flowers, distributed in India, Sri Lanka, China and other southeast Asian countries.[17-18] The roots of this shrubby scandent climber shows a number of circular (annular), variously colored rings in cross section and highly reputed for blood purification.[19-20] *Coccinia indica* also known as Ghilodi in Gujarati and Ivy-gourd in English is a tropical plant in the family Cucurbitaceae. It is an aggressive climbing vine that can spread quickly over trees, shrubs, fences and other supporters.[21-22] *Annona squamosa* also known as Sitapha in Gujarati and Custard apple in English, is a tree occurs in semi-wild conditions in many parts of India. It consists of oblong-lanceolate leaves with entire margin, thin- grey bark, yellowish green syncarpium, fleshy flowers, globose or ovoid light green, fruits with ripe carpels with oblong brownish black seeds.[23-24] In the current study, polyherbal formulation was firstly made with equal ratio of roots of *Salacia prenoides*, aerial parts of *Coccinia indica* and leaves of *Annona squamosa*. Polyherbal formulation was developed from above mentioned diverse ethno-pharmacological application and further microencapsulated by freeze drying method using different wall material Subsequently, Total Phenolic Content, Total Flavonoid Content, antioxidant activity (i.e. DPPH and ABTS+ assays), anti-diabetic potential (i.e., in vitro α-amylase and α-glucosidase inhibition), physical properties like, moisture contents, water activity (a_w), solubility, hygroscopicity, glass transition temperature (T_g), were investigated. In last, the chemo-profiling for ethanolic extract of polyherbal formulation was also studied.

MATERIALS AND MTHODS

Materials, Chemicals, Reagents and Encapsulating Agents

All different parts of herbs (detail in Section 2) were procured from various places of Gujarat and their identification and respective characteristics were authenticated by Prof. Ajay Saluja from A.R.College of Pharmacy and G.H.Patel Institute of Pharmacy, Sardar Patel University, Vallabh Vidyanagar, Anand, Gujarat, India. All chemicals used were of analytical grade or higher where suitable. DPPH (2,2-diphenyl-1-picryl-hydrazyl), Folin-Ciocalteu (FC), Butylated hydroxyltoluene (BHT), TWEEN 20, quercetin, Sodium carbonate, ABTS (2,20-azinobis (3-ethylbenzothiazoline-6-sulphonic acid), α-tocopherol, Linoleic acid, (+)-catechin, quercetin, AlCl3*6H_2O, HCl, Vanilline, NaOH, Potassium persulfate, Trolox, gallic acid were

purchased from Sigma-Aldrich GmbH. α-amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae*, paranitrophenyl-glucopyranoside, pepsin (porcine-7000), bile salts pancreatin (p-1750), piperazine-NN-bis (2-ethane-sulphonic acid) di-sodium salts (PIPES), gelatine (GE), HPLC-grade methanol, acetonitrile, ethanol, acetone were supplied by Sigma-Aldrich, soluble starch (extra pure) was obtained from M.M. Arochem Pvt Ltd, Vile Parle East, Mumbai. Ultra-pure water Milli-Q water (18 MΩ cm^{-1} at 25°C and a TOC value below 5 ppb) was acquired Merck Life Science Private Limited, An affiliate of Merck KGaA, Darmstadt, Germany. Sodium hydrogen carbonate was purchased from Merck (Dermstadt, Germany). Sea sand was of 200-300 grain size from Scharlau (Barcelona, Spain). The encapsulating agent sodium alginate was purchased from (M/S Anurag Trading Co, India) and maltodextrin (Dextrose equivalent of 12) was purchased from Vijaya Entreprises, Mulund West, Mumbai.

Polyherbal Formulation

Polyherbal formulation was made by combining the roots of *Salacia prenoides*, aerial parts of *Coccinia indica* and leaves of *Annona squamosa*, in a ratio of 1:1:1 respectively.

Preparation of Sample

Firstly, the different parts of aforesaid herbs were cut into small pieces, followed by thorough washing with deionised water in order to avoid any contamination. The polyherbal formulation material was then dried for 12 days in dark in well ventilated room at room temperature (23±8°C) and subsequently grounded with mortar and pestle to make crude powder with the help of liquid nitrogen, until a uniform sieve size equivalent to (1.0 mm) was achieved. The resulting powder was stored in deep freeze at -80°C in inert vacuum bags until used for extraction as followed.

Accelerated Solvent Extraction (ASE)

ASE was executed in a Dionex ASE 350 system with the powder of polyherbal formulation obtained as mentioned above. Aliquot of 5.0 g of powder of polyherbal formulation was mixed with diatomaceous earth (1/1) and placed in a 34 ml stainless-steel cells. The extraction was performed via 3 consecutively applied steps with absolute solvents of increasing polarity, in order to get the maximum possible number and amount of secondary metabolites of various polarities and miscibility, namely, acetone, ethanol, methanol and their aqueous mixtures with water (1:10, 3:10) and pure water. Extraction time was of 22 m; pressure 10.6 MPa; temperature 75°C (for acetone, ethanol and methanol) and 135°C (for water). Organic solvents were removed in a rotary vacuum evaporator at 38°C, while the residual water was removed in a freeze drying unit. The extracts after solvent evaporation were placed under nitrogen flow for 20 m and stored in dark glass bottles at -80°C until analyzed.

Development of Matrix type Powder Products

Different combination of encapsulating wall materials was selected as follows and named accordingly: KD_1 (5% SA and 5% MD); KD_2 (5% SA and 5% GE); KD_3 (5% GE and 5% MD) and KD_4 (10% SA). In order to

prepare the particular dispersions, 100 ml of water was mixed with aforesaid polyherbal formulation extract individually with KD_1, KD_2, KD_3 and KD_4, under constant shaking with 220 rpm, at 35°C for 30 m by a shaking instrument (Dolphin Pharmacy Instument Pvt Ltd., Mumbai). Afterwards, these dispersions were microencapsulated through lyophilisation process for formulating four distinctive treatments i.e. KD_1 , KD_2, KD_3 and KD_4. These dispersions were kept strictly at -20°C (deep freezer) for 48 h for encapsulation by means of freeze-drying process.

Subsequently, the samples were placed in laboratory lyophiliser unit (Acmas Technologies Pvt Ltd, India) for freeze drying at -56.5°C, with vacuum pressure of 4.61 mmHg for 60 h. After the completion of freeze drying process, the samples were crushed utilizing a mortar and pestle assembly. Finally, the desirable final encapsulated products were sealed in polyethylene bags and aluminium pouches as well and stored in desiccators encompassing silica until further analysis.

Determination of Bioactive Compounds after Matrix formulation

Total phenolic compounds (TPC) and total flavonoid compounds (TFC) were determined as bioactive components after the microencapsulation of the polyherbal formulation.

Determination of Bioactivities after Matrix formulation

Bioactivities of the microencapsulated powders were measured in terms of total antioxidant activity by $ABTS^+$ [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid] radical scavenging activity and DPPH scavenging activity. All these spectrophotometric analysis were performed according to previously developed methods with minor alteration.[25-26] The results of $ABTS^+$ radical scavenging activity are deliberated as EC_{50} values (mg of extract/mL) for comparison. Effectiveness of antioxidant properties is inversely correlated with EC_{50} value.

Determination of the Physical Properties of the Matrix Powders

Moisture Content

The moisture contents of the encapsulated products were estimated by using the method described in manual AOAC, i.e. by calculating the loss of sample after weight after heating at 105°C.

Water Activity (a_w)

The water activity (a_w) of all lyophilized samples was calculated through the direct analysis in *LabSwift - aw* (a portable water activity unit), to gain the constant state the samples were firstly placed at 25°C for at least 15 m.

Solubility

The solubility of encapsulated products was measured by the method described by *Webb TJ*[27] with minute alterations. The sample's quantity of 1.0 g was mixed up with 100 ml distilled water in beaker and stirred with

magnetic stirrer (MS-H-S10) for 20 m. After that the centrifugation of solution carried out at 3000 × g (Advanced Technocracy Inc. Ambala Cantt, India) for 10 m. The quantity of 25 ml of the supernatant was transferred to a petriplates (pre-weighed) and dried in oven at 105°C for 4.0 h. The solubility was measured as a result of weight difference and demonstrated in the term of percentage (%).

Hygroscopicity

The hygroscopicity of the encapsulated powders was estimated by the method decribed by *Burey P. et. al*[28] with slight vatiation. For the estimation of hygroscopicity, the encapsulated powder of 1.0 g was placed in dessicator with saturated NaCl solution (74.6%) at temperature of 25°C. After 1 week, samples were weighed and hygroscopicity was calculated in the term of percentage (%).

Glass Transition Temperature (T_g)

On heating the polymer to a certain temperature below the heat capacity, plot will shift downward suddenly, means there is more heat flow and there is an increase in the heat capacity of the polymer. This happens because the polymer has just gone through the glass transition and because of this change in heat capacity that occurs at the glass transition, we can use DSC to measure a polymer's glass transition temperature. The glass transition temperature (T_g) of the encapsulated products was calculated by means of temperature modulated differential scanning calorimetry (TMDSC). The weight of 7-8 mg of sample was placed in aluminium hermetic pots. For the reference, an aluminium pan without sample was used. Ultra-pure nitrogen N_2 was used as purge gas (flow rate 50 ml/m). The temperature ranged from -80°C to 120°C at a heating of 40 °C/m. The glass transition temperature was determined by utilizing software of TA Universal Analysis 2000.[29-31]

In vitro Assays

α-amylase Inhibition Assay

The inhibition of α-amylase was determined using an assay modified from the *Hansawasdi C et al* manual. The assay was carried out following the standard protocol with slight modifications.[32] Starch azure (2 mg) was suspended in 0.2 ml of 0.5 M Tris–HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ (substrate solution). The tubes containing substrate solution were boiled for 5 m and then pre-incubated at 37°C for 5 m. Aliquot 0-4 mg/ml in DMSO (v/v 1:1) of each encapsulated PHF samples was prepared and 500 µl of each sample were mixed with 0.2 ml of 0.5 M Tris–HCl buffer (pH 6.9) containing α-amylase solution (0.5 mg/ml) and incubated at 25 °C for 10 m. After pre-incubation, 500 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25 °C for 10 m. The reaction was stopped with 1.0 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 m and cooled to room temperature. The reaction mixture was then diluted by adding 1 5ml of distilled water and the absorbance was measured at 540 nm using a micro-plate reader. The experiments were performed in duplicate and the absorbance of sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were also recorded. The absorbance of the

final each encapsulated polyherbal formulation sample was obtained by subtracting its corresponding sample blank reading. Acarbose was prepared in distilled water and used as positive controls.

The α-amylase inhibitory activity was calculated by using following formula:

$$\% \text{ Inhibition} = \{(Ac-Ae)/Ac\} \times 100$$

Where Ac and Ae are the absorbance of the control and extract, respectively.

The concentration of acarbose and each encapsulated polyherbal formulation required to inhibit 50% of α-amylase activity under the conditions was defined as the IC_{50} value. The α-amylase inhibitory activities of each encapsulated polyherbal formulation and acarbose were calculated and its IC_{50} values were determined by plotting the graph with varying concentrations of the plant extracts against the percent inhibition.

α-Glucosidase Inhibition Assay

The inhibition of α-Glucosidase activity was determined using the modified published method by *Berna Elya et al.*[33] Aliquot of 0-4 mg/ml in DMSO (v/v 1:1) of each encapsulated polyherbal samples were prepared. 50 µl of each concentration sample was mixed well with 100 µl of 0.1 M phosphate buffer (pH 6.9) containing α-Glucosidase solution (1.0 U/ml) and the mixture were then incubated in 96-well plates at 25°C for 10 m. After pre-incubation, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 m. Before and after incubation absorbance readings were recorded at 405 nm using a micro-plate reader and compared to control which contained 50 µL of the buffer solution instead of the polyherbal formulation. The experiments were performed in triplicate and the α-Glucosidase inhibitory activity was expressed as percentage inhibition. Acarbose was prepared in distilled water and used as positive controls.

The percentage inhibition was calculated using the formula;

$$\% \text{ Inhibition} = \{(Ac-Ae)/Ac\} \times 100$$

Where Ac and Ae are the absorbance of the control and extract, respectively.

IC_{50} value (inhibitor concentration at which 50% inhibition of the enzyme activity occurs) of each encapsulated polyherbal formulation samples were determined by plotting graph with varying concentrations of the plant extracts against the percent inhibition.

Acute toxicity

The acute oral toxicity study was carried out in compliance with Organization for Economic Cooperation and Development (OECD) guidelines 423.[34] All mice (n=5) for testing were fasted for 12 h and weigh have been recorded and subsequently received the solution of microencapsulated products of polyherbal formulation at the final concentration of 2000 mg/kg by gavage. The animals were observed individually at least once during the first 30 m after dosing, periodically for first 24 h and regularly thereafter for 14 days for feeding period for gross behaviour changes, toxicity symptoms or mortality.

Qualitative and Quantitative High-Resolution Mass Spectrometry Analysis of ASE

An LC-ESI-Q-TOF-MS apparatus produced by Agilent Technologies (Santa Clara, CA, US) was applied in the study. The instrument was composed of HPLC chromatograph (1260 Series) containing a degasser (G1322A), a binary pump (G1312C), an auto sampler (G1329B), a column oven (G1316A), a PDA detector (G1315D) and a mass spectrometer (G6530B) with quadrupole and a time-of-flight analysers.

For An LC-ESI-Q-TOF-MS analysis, firstly ethanolic extract was prepared using ASE as described in Section 4. Afterwards obtained ethanolic extract was used for the metabolic profiling of polyherbal formulation using an Agilent 1100 Liquid Chromatography system furnished with a standard auto sampler. A gradient of acetonitrile (B) and water (A), both with an addition of formic acid, was carefully adjusted to provide sufficient separation of the extract on a chromatographic column (Zorbax Stable Bond RP-18 Column: 150 mm×2.1 mm, dp=3.5 µm):0 m-1%B in A, 10 m-40%B in A, 12 m-40%B in A, 17 m-95%B in A, 20 m-1%B in A, stop time : 30 m. 0.2mL/min flow rate, 5 min post time, 10 µL injection volume and the *m/z* range from 40 to 1000 were set in the method details. The analysis was performed at 25°C on a freshly calibrated apparatus. Two most intensive signals were simultaneously fragmented in the MS/MS analysis. After the collection of 1 spectrum, they were excluded for 0.3 min from the analysis to collect more fragmentation data from other less intense signals. A calibration mixture was dosed simultaneously during the analysis as an internal standard to sustain high accuracy of the measurements. The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to compound identification. By using this method, an exact calibration curve based on several cluster masses, each differing by 82 Da ($NaC_2H_3O_2$) was obtained. Due to the compensation of temperature drift in the microOTOF-Q II, this external calibration provided accurate mass values of better than 5ppm for a complete run without need for a dual sprayer setup for internal mass calibration.

Statistical Analysis

All statistical analyses were conducted using a one-way analysis of variance using Dunnett's comparison tests or unpaired t-tests. All calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Significance was observed at $p < 0.05$.

RESULTS AND DISCUSSION

The consequence of encapsulation of the Contents of Antioxidant Components of Polyherbal formulation

The contents of antioxidant components of polyherbal formulation extract treated with different encapsulating wall materials were shown in Table 1. In comparison to untreated extract, all encapsulated treatments have less antioxidant components (i.e. TPC and TFC). The retention for all freeze-dried treatments demonstrated in the term of percentages, ranged from 95.29% to 69.23% for TPC and 77.47% to 41.36% for TFC representing the effectiveness of encapsulation procedure. The wide-ranging powders produced from the matrix type encapsulation process, especially those obtained from KD_4, retained higher contents of antioxidant

components. In general, these results may be associated with the type and concentrations of different wall materials.

Table 1. Antioxidant components of Polyherbal formulation extracts encapsulated with Sadium alginate (SA), gelatine (GE), Maltodextrin (MD) and their combinations by Freeze-drying method.

Treatment	Total Phenolic Content[1] (TPC)	Total Flavonoid Content[2] (TFC)
Control	27.73±0.62	6.858±0.06
KD_1	25.27±0.095	4.193±0.08
KD_2	23.90±0.42	3.827±0.04
KD_3	19.28±0.16	2.770±0.04
KD_4	26.27±023	5.243±0.16

Note: Results displayed are a representation of triplicate quantification per extract. KD_1: Freeze-dried, with 5% SA and 5% MD; KD_2: freeze dried with 5% SA and 5% GE; KD_3: freeze dried with 5% GE and 5% MD; KD_4: freeze dried with 10% SA. Total phenolic contents[1] (TPC) expressed as mg gallic acid equivalents (GA E) per g of dry extract, Total flavonoid content[2] expressed as mg quercetin equivalent (Q E) per g of dry extract. The Dunnett's test was to evaluate the significance with confidence level was set to 95%; different letters within the same column indicate significant differences (p <0.05)

There were many multifaceted factors which were responsible for hammering of polyphenol compound during freeze drying method, the crushing of lyophilized encapsulated products after freeze-drying, were considered one of the key factors which may cause the degradation of bioactive components in the final products by boosting the product's contact with environment. Other factors which may responsible for declining the concentration of active components include: formation of microspheres during the lyophilisation due to a scattering of the bioactive components inside the configuration of encapsulating wall materials i.e., consisting of one or more constant phase of encapsulating agents,[35] development of micro-pores in the aforesaid microspheres, mainly associated to sublimation process during lyophilisation.[36] In the current study, lyophilized product encompassed a reduction of 6.73%-32.79% for TPC and declined trend of 24.55-60.66% and was also observed for TFC. Despite the reduction of antioxidant components of encapsulated products, a significant retention was also observed with Sodium Alginate (SA) having phenolic retention of 95.11%.

The freeze dried product encapsulated with 10% SA (KD_4) demonstrated the exceptional conservation for antioxidant components (i.e. TPC and TFC). The order of effectiveness of microencapsulation for other remaining treatments was as followed: $KD_1 > KD_2 > KD_3$. The higher competence of KD_4 treatment was mainly attributed to the structure of SA, because it is a hetero-polymer made up of dense branches of sugar, containing a minute quantity of protein which connected to the carbohydrate skeleton via covalent bonds, proceeding as a tremendous encapsulating material.[37] Noteworthy results were also found for KD_2, which might be credited to presence of 5% SA. In contrary, no significant difference was noticed for the lyophilized product having 5% GE and 5% MD (KD_3).

Antioxidant activity of the encapsulated contents of the antioxidant components of polyherbal formulation

The antioxidant activity of encapsulated powders determined by DPPH and $ABTS^{+}$ assay as illustrated in Table 2.

Table 2 Antioxidant activities of Polyherbal formulation extracts encapsulated with Sadium alginate (SA), Gelatine (GE), Maltodextrin (MD) and their combinations by Freeze-drying method.

Treatment	DPPH[3]	ABTS[4]
Control	134.3±1.80	3.697±0.03
KD_1	86.01±0.5	3.207±0.95
KD_2	75.74±4.61	2.783±0.07
KD_3	52.53±0.73	2.295±0.082
KD_4	79.12±1.68	2.728±0.135

Note: Results displayed are a representation of triplicate quantification per extract. KD_1: Freeze-dried, with 5% SA and 5% MD; KD_2: freeze dried with 5% SA and 5% GE; KD_3: freeze dried with 5% GE and 5% MD; KD_4: freeze dried with 10% SA. DPPH[3] expressed as µmol/gm sample on dry basis; EC_{50} (mg/mL) is representative of the effective concentration at which 50% of ABTS+[4] radicals were scavenged. The Dunnett's test was to evaluate the significance with confidence level was set to 95%; different letters within the same column indicate significant differences (p <0.05)

All matrix type encapsulated products had showed decrease antioxidant activity by DPPH assay in relation to original extract (control) and their retention ranged from 39.85-65.50%. . KD_1 (5% SA and 5% MD) and KD_4 (10% SA) illustrated the highest antioxidant activity; these results were agreement with previously found values by Souza et al.[38] The order of effectiveness was noticed as: KD_1>KD_4>KD_2>KD_3. In the case of DPPH assay, the antioxidant retention for all encapsulated products were explored from 78.60% to 94.94% in comparison to original extract and KD_1 (5% SA and 5% MD) showed maximum value for antioxidant activity. Remaining treatments have been categorized in context of efficacy as followed: KD_4>KD_2>KD_3. Referring to antioxidant assay by ABTS+ radical scavenging activity, the range of retention was from 63.21% to 87.69%. The noteworthy consequence was revealed for KD_1 (5% SA and 5% MD), while KD_2 (5% SA and 5% GE) and KD_4 (10% SA) also illustrated the significant results with retention of 76.28% and 75.19% respectively. The above discussion suggested the worthiness of diverse antioxidant assay for secure and overwhelming conclusion, because each assay comprised its own preciseness and proceeds at a challenging site of action. Amongst the all lyophilized encapsulated products, the antioxidant activity was higher in KD_1 and KD_4, being related to the presence of high antioxidant components (i.e. TPC and TFC) (Table 1), which provided an excellent defence system against unrestrained oxidation, owing to its high reducing power.

Physical Characteristics of Matrix type encapsulated Powder Products

Physical characteristics i.e., water activity; moisture contents and hygroscopicity are indispensable for encapsulating products steadiness and storage, whilst aqueous solubility is correlated with ability of powder products for reconstitution.[16]

The moisture contents for four different lyophilized encapsulated products were demonstrated in Figure-1. The moisture content of said powders were ranged from 7.07% to 9.04%; on the contrary, no significant difference was found between KD_2 and KD_3 (7.41% and 7.21% respectively).

The water activity (a_w) of all encapsulated product (Figure-2) was ranged from 0.310 to 0.450 and all final encapsulated products were noticeably dissimilar from one another, apart from KD_1 (5% SA and 5% MD). KD_4 (10% SA) demonstrated the maximum water activity (a_w) value of 0.450 which was corroborating with previous study carried out by *Labuza T.P. et al*.[39]

Solubility of matrix type encapsulated powder product was determined by various factors such as: the feed composition and particle size. The selection of the wall material is very important, not only for the solubility itself but also to the crystalline state that ultimately bestowed to the dried powders.[40] The aqueous solubility for all lyophilized treatments was ranged from 84.06% to 92.31% as illustrated in Figure-3. The solubility of the final product possibly not only associated with solubility prospective of encapsulating wall material but also on attainted particle size in final desirable product; if particle size would be minute, it would ultimately provide the better surface area's availability for the hydration process.[41-42] The highest solubility value was obtained for treatment KD_4 (10% SA) which was consistent with previous work.

Figure-1 Moisture Content

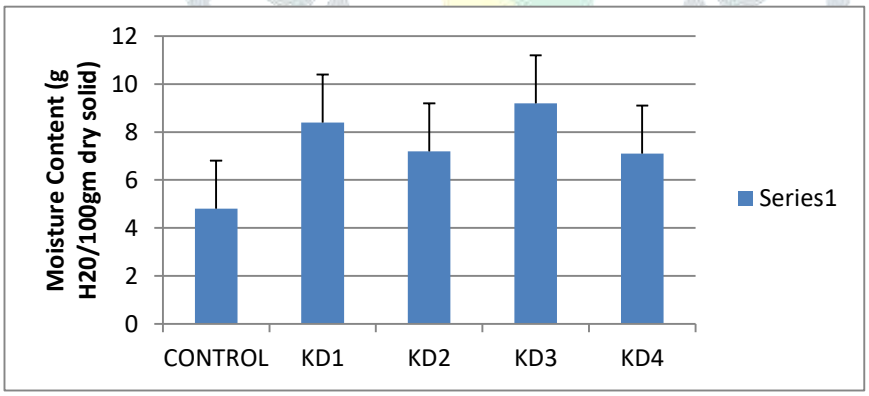


Figure-2 Water Activity (a_w)

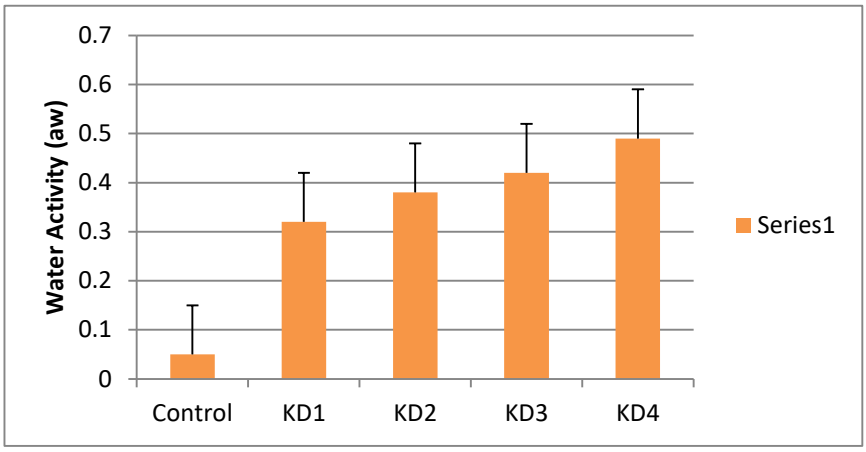
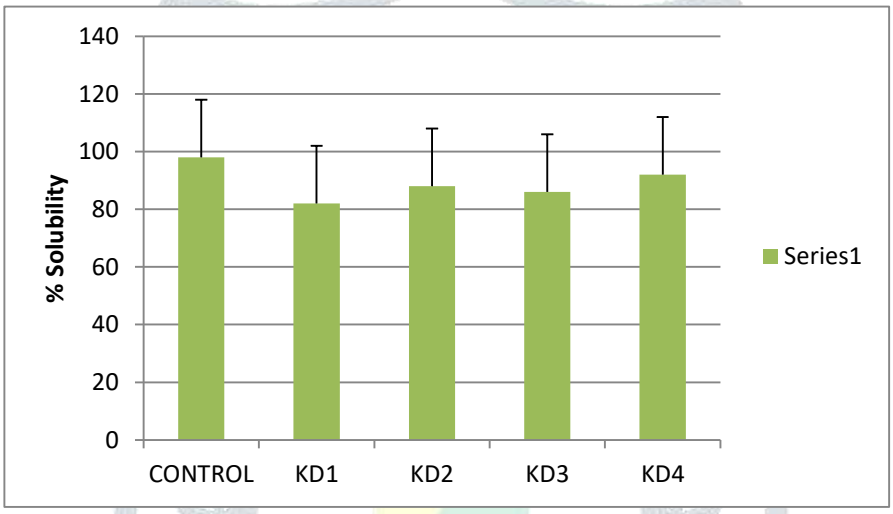
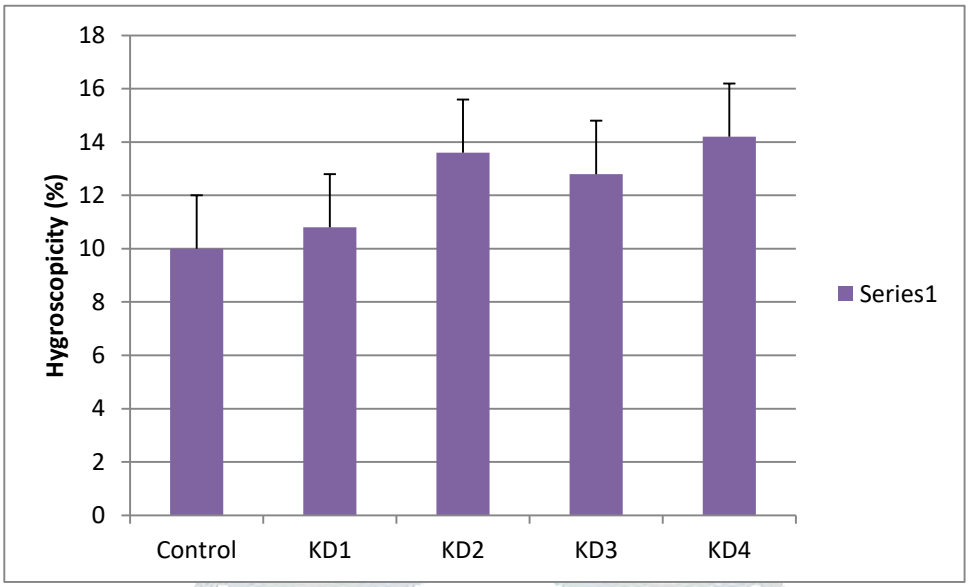


Figure-3 Solubility (%)



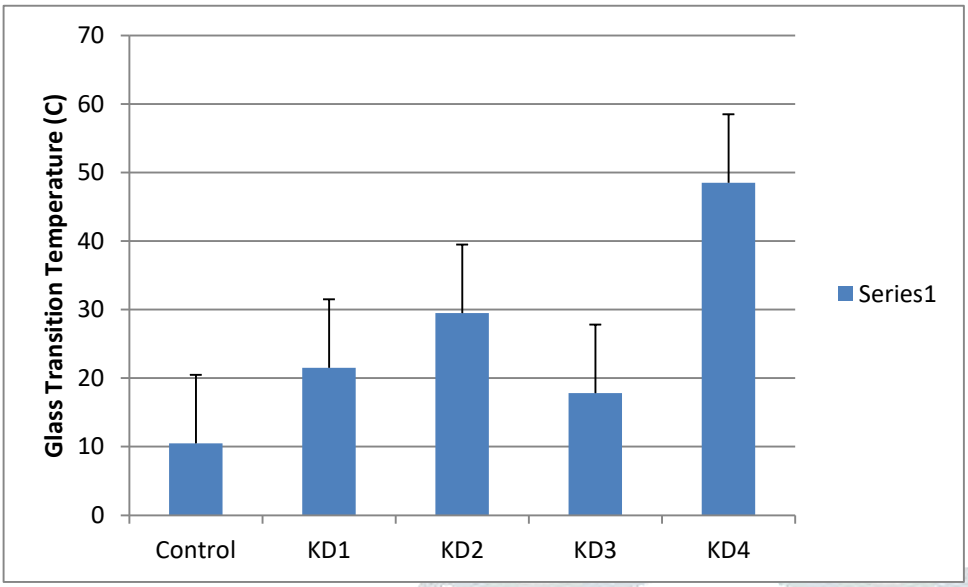
The hygroscopicity values for all encapsulated powder products by the means of freeze-drying method were depicted in Figure-4. These were ranging from 11.92% to 14.35%, representing a lesser amount of hygroscopicity values for powder products hence assisted the protection of antioxidant components. The findings of current work have much resemblance with preceding work, utilizing related sort of encapsulating wall materials. The lyophilized powdered products demonstrated the lesser hygroscopic values, regardless the presence of higher contents of moisture.[12] The lower values of hygroscopicity for the all lyophilized products mainly attributed to the bigger particle size, since the bigger the particle size, the lesser the uncovered surface area, therefore low down the water absorption.[16,43]

Figure-4 Hygroscopicity values



The stability of encapsulated powdered products for the period of storage was principally determined by glass transition temperatures (Tg), the lower the Tg resulting in lower the stability of final product and vice versa. The glass transition temperature of all lyophilized products was of 16.87°C to 45.0°C in range (Figure-5). Amongst all lyophilized matrix type encapsulated products, the KD_4 represented the highest glass transition temperature (45.0°C), proving maximum stability. Furthermore, other treatments also showed significant values for Tg except KD_1 and KD_3. The glass transition temperature has been influenced by diverse factors, including moisture contents, chemical configuration and molecular mass of subject matter.[44] Additionally, integration of encapsulating agents in extracts has much predisposed on glass transition temperatures which varied accordingly to molecular weight of encapsulating material; increase in molecular weight of wall material resulting the increase in final Tg of the product. The lyophilized matrix type encapsulated product obtained from treatment KD_1 and KD_3 represented the lower Tg because of lower molecular weight of MD. Moreover, this behaviour was not noticed in KD_2 and KD_4 due to existence of uppermost molecular weight of SA in the term of quality and quality of wall material.

Figure-5 Glass Transition Temperature



α-Amylase and α-Glucosidase inhibition

Type-II DM an outcome of insulin resistance is a metabolic disease, that according to the latest data for the World Health Organization in 2014, impinges on 9% of the world's population, both in developed and developing countries and directly caused 1.5 million deaths in that single year.[45-46] In order to hamper the side effects of type-II DM, insulin injection and usage of anti-hyperglycaemic substances are two key conventional approaches. The management of the blood sugar level is effective and novel approach to overcome the diabetes mellitus and related complications. Inhibitors of carbohydrate hydrolysing enzymes (i.e., α-amylase and α-glycosidase) have been practically valuable as oral hypoglycaemic drugs and regarded as a reliable indicator for the efficacy of therapeutic agents.[47] Several α-amylase inhibitors including acarbose, miglitol and voglibose are clinically useful to treat diabetes but these are expensive and have considerable clinical side effects. Medicinal plants have great potential to retard the absorption of glucose by inhibiting the saccharides hydrolysing enzymes.[48-49] There was an attempt to explore the remarkable drugs from medicinal plants featured with elevated potency and less adverse effects than existing drugs.[50] Therefore, screening and isolation of inhibitors from plants for these enzymes are escalating.

In the aforementioned context, our matrix type encapsulated polyphenolic enriched powders were investigated for α-amylase and α-glycosidase inhibition as shown in Figure-6 and Figure-7. Diverse classes of polyphenolic compounds in the current polyherbal formulation extract were detected likewise: flavonoids, alkaloids, terpenoids, lignans, glycerophospholipid, prenol lipids and their derivatives, which eventually may be considered for anti-diabetic potential of matrix type encapsulated powders of current study. The treatment KD_1 (5% SA &and 5% MD) demonstrated the highest inhibition at concentration 4 mg/mL, for α-amylase (94.34±2.66, with IC50 value 1.48 mg/mL ±0.58) and α-glucosidase (74.40±1.65 with IC50 value 2.03±0.46 mg/mL), representing highest anti-diabetic potential. Previously, none of investigation has yet been carried out on lyophilized aforementioned encapsulated polyherbal formulation products.

Figure-6 α-amylase inhibition

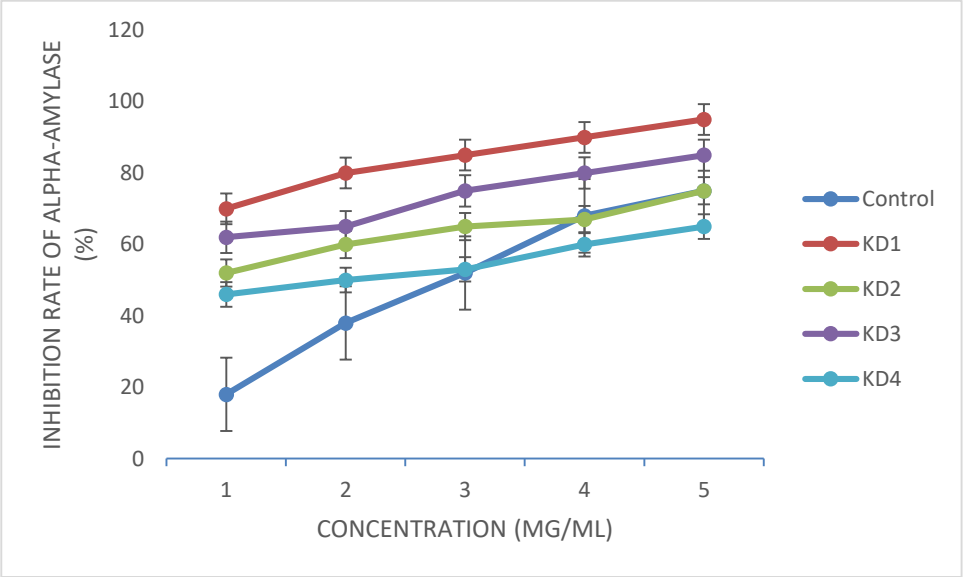
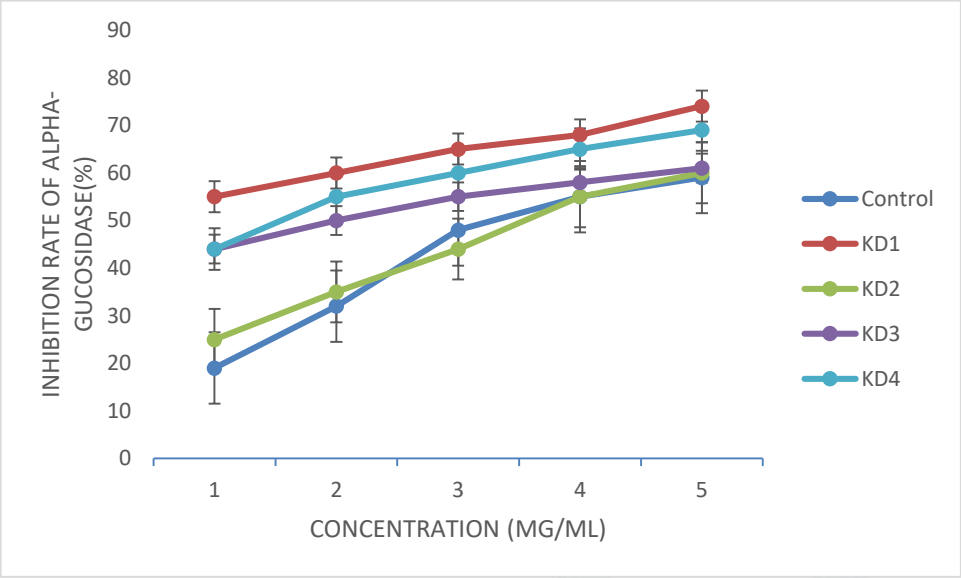


Figure-7 α-glucosidase inhibition



Acute Toxicity

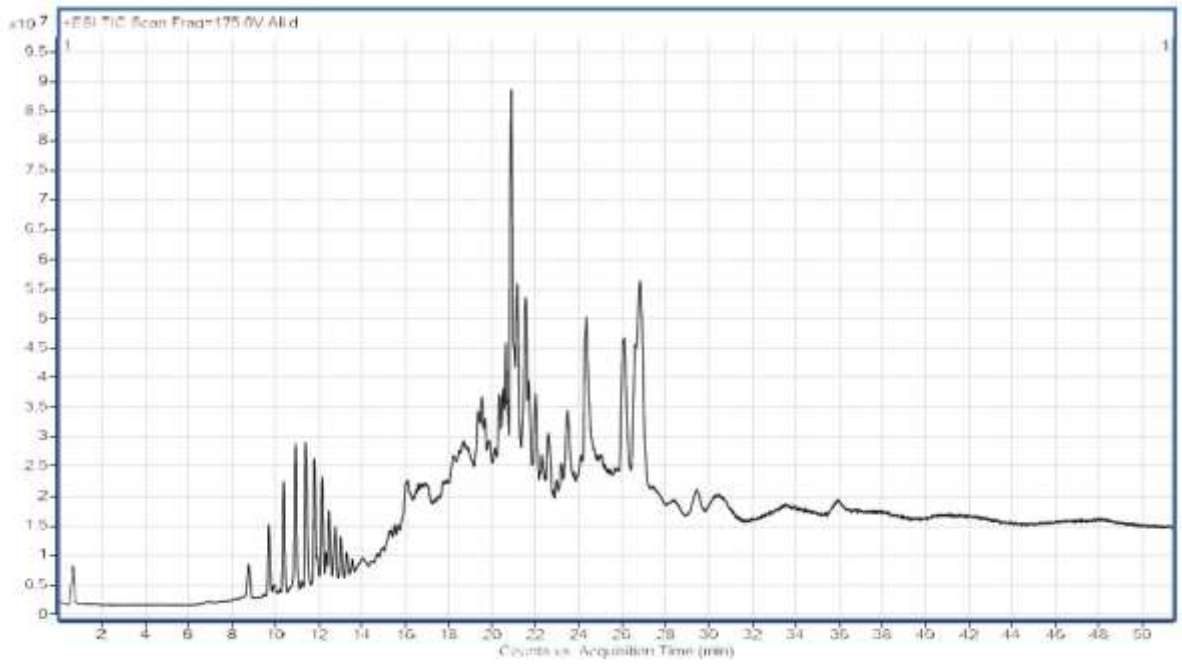
No toxic effects and mortality were observed at a dose of 2000 mg/kg by gavage. Consequently, encapsulated products of polyherbal formulation extract were regarded as safe for consumption.

Bioactive compounds from High-Resolution Mass Spectrometry Analysis of ASE

The ethanolic extract of freeze dried fine powder of polyherbal formulation was a multifaceted mixture of compounds. Figure 8 characterized the chromatogram of said ethanolic extract. The bioactive compounds were recognized by means of the comparing retention times (RT) and MS/MS spectra granted by QTOF-MS with those of valid standards wherever obtainable and via elucidation of MS and MS/MS spectra from QTOF-MS merged with data available in literature. MS data of identified compounds has been recapitulated in the

table 3 including calculated *m/z* for molecular formula provided, main fragment obtained by MS/MS, error and proposed compound for each peak. Diverse classes of polyphenolic compounds have been discovered in the ethanolic extract of polyherbal formulation. Annotated compounds represented the diverse classes includes flavonoids, alkaloids, terpenoids, lignans, glycerophospholipid and prenol lipids.

Figure-8 Chromatogram of the Ethanolic extract derived from freeze dried powder of Polyherbal formulation



Peaks 5,8,9,13,14,15,16,17,31,32,33,38,42,45 and 46 represented different flavonoid compounds and their derivatives which possess diverse biological activities which were proven previously i.e., anti-oxidative, anti-nociceptive, anti-diabetic.[51-53] Among them 3 bioactive compound (peak 9, 17 and 38) were classified as 6-prenylated flavones (i.e., flavones that features a C5-isoprenoid substituent at the 6-position). These bioactive compounds are insoluble in aqueous solution and designated as a faintly acidic compound. These compounds previously found in fruits, peas and pulses and considered to be flavonoid lipid molecule. While some compounds (peak 16, 42 and 45) belong to sub class flavonoids glycosides likewise; quercetagetin 7-glucoside (compound 16, *m/z* 481.3673 [M + H]) and quercetin 3-(6'-malonylglucoside)-7-glucoside (Compound 42, m/z 713.62 [M+H]) were recognized as flavonoid-7-o-glycosides. These are phenolic compounds containing a flavonoid moiety which is *O*-glycosidically linked to carbohydrate moiety at C 7-position. These derivatives of flavonoids have priory proved strong antioxidative, anticancer, neuro-protective, anti-inflammatory, diuretic and hypoglycaemic activities. Moreover, catalpol *m/z* 363.195 [M+H] (compound 15) demonstrated a variety of biological activities including anti-cancer, neuro-protective, anti-inflammatory, diuretic, hypoglycaemic and anti-hepatitic virus effects. Previously studies have also provided some clues that catalpol can affect energy metabolism through increasing mitochondrial biogenesis, enhancing endogenous antioxidant enzymatic activities and inhibiting free radical generation ultimately attenuates oxidative stress.[54]

Table-3 Bioactive Compounds identified in Ethanolic Extract of Polyherbal formulation

Peak No.	RT (min)	Assigned Compound Name	Elemental Composition	m/z $[M+H]^+$	Difference (mDa)
1	8.69	Kotalagenin 16-acetate	$C_{32}H_{50}O_5$	514.747	0.63
2	9.49	Salacinol	$C_9H_{18}O_9S_2$	334.354	-0.89
3	9.695	Proanthocyanidin A	$C_{31}H_{28}O_{12}$	592.553	0.21
4	9.912	Leucopelargonidin	$C_{15}H_{14}O_6$	290.079	-0.18
5	9.96	Cathinone	$C_9H_{11}NO$	149.193	1.05
6	10.181	Zanthobisquinolone	$C_{21}H_{18}N_2O_4$	407.184	0.61
7	10.371	Dulcitol	$C_6H_{14}O_6$	182.172	0.67
8	10.641	Mangiferin	$C_{19}H_{18}O_{11}$	422.342	0.42
9	10.752	Alabin D	$C_{25}H_{26}O_5$	407.184	0.95
10	10.829	3-*O*-rutinoside (rutin)	$C_{27}H_{30}O_{16}$	610.521	0.28
11	10.918	Myricetin	$C_{15}H_{10}O_8$	319.1692	0.4
12	10.942	16-hentriacontanone	$C_{31}H_{64}$	436.853	0.65
13	11.198	β-sitosterol	$C_{29}H_{50}O$	414.718	0.92
14	11.391	2,3-trans-3,4-cis-flavan-3-ol	$C_{15}H_{14}O_2$	226.270	-0.17
15	11.458	euonysterol	$C_{31}H_{52}O_2$	455.98	0.9
16	11.497	*n*-octacosanol	$C_{28}H_{58}O$	410.76	0.51
17	11.798	Quercetin -3-*0*-β-D-galactoside	$C_{15}H_{10}O_7$	302.236	-0.03
18	11.951	Tingenone	$C_{28}H_{36}O_3$	420.593	-0.18
19	12.159	Nonfriedelane triterpene	$C_{30}H_{48}O_7S$	552.767	0.9
20	12.350	Isomultiflorenol	$C_{30}H_{50}O$	426.729	0.08
21	12.471	Butyrospermol	$C_{30}H_{50}O$	426.729	-1.93

22	12.76	Ent-kaurane diterpenoid	$C_{20}H_{32}$	272.468	0.93
23	13.051	24-methylenecycloartenol	$C_{31}H_{52}O$	440.756	0.72
24	13.280	(24R)-ethylcholest-5-en-3β-ol glucoside	$C_{28}H_{46}O$	398.68	-1.2
25	13.571	Annomosin A	$C_{40}H_{60}O_5$	620.444	-0.25
26	16.111	Annosquamosine C	$C_{19}H_{32}O_2$	292.463	-0.06
27	17.061	(+)-*O*-methylarmepavine	$C_{20}H_{25}NO_3$	327.417	0.72
28	19.541	Tingenine B	$C_{28}H_{36}O_4$	436.261	0.21
29	20.651	3β,22 β-dihydroxyolean-12-en-29-oic acid	$C_{30}H_{48}O_4$	472.355	-0.3
30	20.882	PA *(18:3(6Z,9Z,12Z)/20:3 (8Z,11Z,14Z)*	$C_{41}H_{69}O_8P$	721.4644	0.32
31	21.150	*Trans*-ocimene	$C_{10}H_{16}$	136.238	0.34
32	21.549	24-methylene-24-dihydrolanosterol	$C_{33}H_{56}O_2$	482.793	1.24
33	21.701	β-caryophyllene	$C_{15}H_{24}$	204.357	0.18
34	22.021	desmosterol	$C_{27}H_{44}O$	384.648	0.44
35	22.384	3-Hydroxyolean-12-en-28-oic acid	$C_{30}H_{48}O_3$	456.711	0.57
36	22.591	2',5,6-trimethoxyflavone	$C_{18}H_{16}O_5$	312.321	1.52
37	22.621	Ubiquinol-8	$C_{49}H_{76}O_4$	729.507	0.29
38	22.653	Luteone	$C_{20}H_{18}O_6$	354.37	0.19
39	22.978	Salaquinone A	$C_{28}H_{34}O_5$	450.567	1.59
40	23.184	Norlaureline	$C_{18}H_{17}NO_3$	295.338	1.38
41	23.529	Quercetin 3-(6"-malonyl glucoside)-7-glucoside	$C_{30}H_{32}O_{20}$	712.566	0.57

42	24.349	β-farnesene	$C_{15}H_{24}$	204.357	0.43
43	26.095	Epigallocatechin 3,3'-di-*O*-gallate	$C_{29}H_{22}O_{15}$	610.179	0.56
44	26.820	Kaempferol 3-(2"-3"-diacetyl-4"-*p*-coumaroylrhamnoside)	$C_{34}H_{30}O_{14}$	662.6	0.34
45	29.447	Quercetin 3-*O*-rutinoside (rutin)	$C_{21}H_{20}O_{12}$	464.0955	0.24

Flavonoids and other polyphenols exhibited the highest inhibitory activity when assayed for the ability to scavenge the diphenylpicrylhydrazyl (DPPH) free radical. Mangiferin (peak 8, m/z 422.342[M+H]) improves cholesterol by activation of PPAR-α (PPAR-alpha) receptors. Mangiferin obtained from *Salacia prenoides* extract inhibit the enzyme α-glucosidase. This enzyme in the digestive tract break down foods into smaller sugars that can be absorbed by the body. If these enzymes are blocked, it takes longer to absorb the sugars.[55] Salacinol (peak 2, m/z 334.354[M+H]) and kotalagenin 16-acetate (peak 1, m/z 514.747[M+H]) lowers blood sugar, triglycerides and cholesterol and they prevent heart hypertrophy by blocking angiotensin response and decreasing certain protein synthesis. A non-friedelane type triterpene Salaquinone A (peak 39, m/z 450.567[M+H]) was elucidated on the basis of chemical and physiochemical evidence. In addition 3β, 22 β-dihydroxyolean-12-en-29-oic acid (peak 29, m/z 472.355[M+H]) and Tingenine B (peak 28, m/z 436.261[M+H]) were found to show an inhibitory effect on rat lens aldose reductase.[56]

Among the known natural bioactive compounds, terpenoids are considered to be approximately 55%. Plant terpenoids are extensively used for their aromatic qualities and play a role in traditional herbal remedies, for instance; a new ent-kaurane diterpenoid, Annomosin A (peak 25, m/z 620.444[M+H]) was identified as peak 25 with molecular mass 620.444 and this compound was identified by Yang Y.L. et al who also identified 14 known ent-kaurane diterpenoids.[57] The 95% ethanolic extract of poly herbal formulation containing *Annona squamosa* leaves found to yield Quercetin -3-*0*-β-D-galactoside (peak 17, m/z 302.236[M+H]) known as hyperoside and Quercetin 3-*O*-rutinoside (rutin) (peak 45, m/z 464.0955[M+H]) known as rutin found to have good antioxidant activity.[58] Mass spectroscopy revealed the presence of β-caryophyllene (peak 33, m/z 204.357[M+H]) as the major constituent. 16-hentriacontanone (peak 12, m/z 436.853 [M+H]) and β-sitosterol (peak 13, m/z 414.718[M+H]) were purified, characterized and evaluated for their antibacterial activity against *Staphylococcus aureus*, *Staphylococcus albus* and *Streptococcus uridans* (all gram +ve bacteria) and *Escherischia coli*, *Pseudomonas pyocyanea* and *Klebisella* (all gram -ve bacteria).[59] Alabin D (peak 9, m/z 407.184[M+H]) from the leaves of Annona squamosa was estimated for Total Antioxidant Activity (TAA) by ABTS/$K_2S_2O_8$ discoloration method.[60]

Lignans were usually found in fruits and have proved strong anti-cancer and antioxidant activities. Among them, compound Euonysterol (peak 15, m/z 302.236[M+H]) and Isomultiflorenol (peak 20, m/z 426.729[M+H]) belong to class amorphous bitter principle and a pentacyclic triterenoid that is oleanan-3-o

lacking the methyl group respectively. It is present in number of plants including *Coccinia indica* aerial parts.[61] Besides this, peak 42 and peak 37 represented sesquiterpene and polyprenyl substituent of octaprenyl. Ubiquinol is the reduced form of Coenzyme Q which is an effective anti-oxidant.

Other detected compounds which were not discussed in detail such as peak 3, 4, 11, 16, 31, 40 are intermediate products of either metabolism or biosynthesis of amino acids, lipids or others. For instance, metabolite 3 represented a polyphenol found in variety of plants. Chemically, they are oligomeric flavonoids. Many are oligomers of catechin and epicatechin and their gallic acid esters. Peak 4 represented Leucopelargonidin which is a colorless compound related to leucoanthocyanins. Peak 11 is member of the flavonoid class of polyphenolic compounds, with antioxidant properties which is commonly derived from vegetables, fruit, nuts and berries. Compound 30 is the phosphatidic acid, produced in glycerolipid biosynthesis. The existence of such compound is mainly attributed to the seeds of *Coccinia indica*. The 31st peak recognized as Trans-ocimene which is an isomeric hydrocarbon. The ocimenes are monoterpenes found within a variety of plants and fruits.

As can been concluded that the current polyherbal formulation is the mixture of previously proven health promoting herbs' parts such as *Salacia prenoides*, *Annona squamosa* and *Coccinia indica*, so diversity and abundance of such detected antioxidant substances/metabolites not only made sense but also verify the outcomes. Taking together, this is the first study which exploited the metabolite profiling of said polyherbal formulation enriched with antioxidants and their evaluation for bioavailability and anti-diabetic potential after encapsulation.

CONCLUSION

In the current study, polyherbal formulation polyphenolic extract was encapsulated by utilizing maltodextrin (MD), sodium alginate (SA) and gelatin (GE) as encapsulating wall materials, due to which resulting capsule product found to have withholding capacity of Total Phenolic Content more than 95.29% except KD_3 (69.23%), while conserving range of Total Flavonoid Content was found near to77.470% except KD_3 (41.36%). Elevated antioxidant activity was also revealed for KD_4 and KD_1 and reasonable for KD_2 and KD_3, representing noteworthy and positive correlation of antioxidant assays to all aforementioned antioxidant components. Taking all results into consideration, KD_4 (10% SA) and KD_1 (5% SA and 5% MD) showed the best performance attributable with respect to the higher preservation of antioxidant components and antioxidant activity by means of DPPH and significant for an $ABTS^+$ radical scavenging activity, augmented by low contents of moisture, water activity (a_w), particle dimension and elevated solubility, hygroscopicity and T_g. Additionally, the aforementioned treatments also demonstrated the excellent morphological features with asymmetrical (irregular) micro-particle structures, depicted lower prevalence of coarseness and crankiness. Moreover, KD_1 and up to certain extent KD_2 and KD_4 also characterized the highest anti-diabetic potential by reason of their significant inhibition rate for α-amylase and α-glucosidase. In addition, no mice proved any toxicity sign at a dose of 2000 mg/kg by gavage for any treatment. In the conclusive manner, we recommended the KD_1 and KD_4 as result of their incredible capability for preserving antioxidant components

to its usage in nutraceutical and functional products while masking the undesirable flavour distinctiveness of herbs/herbal extracts.

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CONFLICT OF INTEREST

None.

SUMMARY

Matrix type encapsulated polyherbal formulation was developed using freeze dried extracts of *Salacia prenoides*, *Annona squamosa* and *Coccinia indica*. This polyherbal formulation was evaluated in-vitro for its antidiabetic potential and found to show very impressive results in terms of stability and efficacy studies.

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