Original Research Article

PHARMACOPEIAL TESTING OF POLY-HERBAL FORMULATION FOR THE MANAGEMENT OF DIABETES MELLITUS

Abstract

Diabetes mellitus is a global cause of morbidity and mortality. There is a continuous rise in the patients of diabetes in Pakistan. Many conventional and natural origin medicines are available for maintaining optimum blood sugar level. There is a need to carry out standardization of the natural origin medicine to authenticate their efficacy and safety.

The objective of this research work was to carry out preliminary pharmacognostic, phytochemical, biological, pharmacological studies on poly-herbal formulation.

The poly-herbal preparation was prepared and evaluated for the standard pharmacopeial tests. The results of the tests performed were found to be adequate to evaluate the poly-herbal formulation and may be used as reference standards in future for further studies.

Key Words: Anti-Microbial Activity, Fourier transform Infra-red spectroscopy, Microscopic, Organoleptic, Thin-layer chromatography

1. INTRODUCTION

Diabetes mellitus is a rising socio-economic burden globally. Pakistan is one of the 19 countries and territories of the International Diabetes Federation, Middle East and North Africa (IDF MENA) region. Worldwide, 425 million people have diabetes. In MENA region more than 39 million people suffer from diabetes and by 2045, the figure will rise to 67 million [1]. There are many herbs with proven anti-diabetic activity. The brief description of the herbs included in the formulation is given below:

Syzygium cumini

Syzygium cumini (Linn.) Skeels (Myrtaceae) is commonly known as Indian blackberry; Jaman. The fruits are rich in raffinose, glucose, fructose, citric acid, mallic acid, gallic acid, anthocyanins; delphinidin-3-gentiobioside, malvidin-3-laminaribioside, petunidin-3-gentiobioside, cyaniding diglycoside, petunidin and malvidin. Black plum fruit and its leaves are good for diabetic patients. The black plum has anti-diabetic features. The fruit helps to convert starch into energy and keep your blood sugar levels in check [2].

Momordica charantia

Momordica charantia (bitter melon or bitter gourd) is a flowering vine in the family Cucurbitaceae. The main constituents of bitter melon which are responsible for the antidiabetic effects are triterpene, protein, steroid, alkaloid, inorganic, lipid, and phenolic compounds. It is a popular plant used for the treating of diabetes. *M. charantia* has significant antidiabetic as well as hypolipidemic activity so that it can be used as an adjuvant along with allopathic treatment of medicine to treat diabetes as well as to delay the late complications of diabetes [3].

Wrightia tinctoria

Wrightia tinctoria (Indarjou, pale indigo plant) belongs to family Apocynaceae. The chemical constituents of *W. tinctoria* includes lipid, saponin, tannin, alkaloid, phenol, steroid, and flavonoid. W. tinctoria possess significant anti-diabetic activity [4].

Gymnema sylvestre

Gymnema sylvestre (Asclepiadaceae), popularly known as "gurmar" for its distinct property as sugar destroyer. The major constituents' saponins, alkaloids, anthraquinones, flavones, hentriacontane, pentatriacontane, phytin, resins, tartaric acid, formic acid, butyric acid, lupeol, β-amyrin related glycosides, stigmasterol, and calcium oxalate. The phyto-constituents responsible for sweet suppression activity includes triterpene saponins known as gymnemic acids, gymnemasaponins, and a polypeptide, gurmarin. G. sylvestre has prospective clinical data in support of treatment of diabetes as it displays favorable effects on blood sugar homeostasis, controls sugar cravings, and promotes regeneration of pancreas [5].

2. Material & Method

2.1. Plants

The medicinal plants; *Syzygium cumini*, *Momordica charantia*, *Wrightia tinctoria***,** and *Gymnema sylvestre* were procured from local pansare and authenticated. All the procured and authenticated individual drugs were dried in shade and cleaned by hand sorting. The individual drugs were then crushed using grinder and passed through mesh no. 40. The individual drugs were then weighed as per the quantity required. The drugs were mixed. The mixed formulation was unloaded, weighed, and packed in labeled glass bottles.

2.2. Chemicals

All the chemicals and reagents were procured from the authorized dealer (Merck, Germany; BDH Chemicals England and Sigma-Aldrich, USA).

2.3. Extraction procedure

The fine powdered drug was macerated with ethanol and kept for 15 days at room temperature for percolation. The ethanol extract was then filtered. This procedure was repeated thrice. Ethanol from extract was evaporated under reduced pressure in a rotary evaporator to obtain an extract.

3. EXPERIMENTAL PROCEDURES

3.1. MICROSCOPIC EVALUATION

Microscopic evaluation was carried out using scanning electron microscope (SEM) [6].

3.2. POLY-HERBAL POWDERED DRUG FORMULATION REACTIONS WITH CHEMICALS

Powder sample was tested phyto-chemically reacting with different chemical agents and triturate with different chemical agents to check the stability, chemical and physical property of the sample with the used chemical agents. Chemical agents; ethyl acetate, HCL acetone, benzene, H_2SO_4, 66%, 5% NaOH 100% H_2SO_4, nitric acid, 5%$FeCl_3$ were used and triturated with water [7].

3.3. PHYTO-CHEMICAL IDENTIFICATION TESTS OF POLY-HERBAL FORMULATION

The following tests are performed to identify chemical constituents present in herbals. Benedicts test for carbohydrate, modified Borntrager's test for Anthrol glycoside, Molish test for carbohydrates, Liebermann's burchard's test for sterols, Froth test for Saponins, Foam test for Saponins, Salkowski test for Sterols, Glycerin test for Tannins, Xanthoprotic test for Protein, Ferric chloride test for Phenols, Alkaline reagent test for Flavonoids, Fehling test A and B, Lead acetate test for Flavonoids, Wagner test, Hager test, has been performed to evaluate the properties of drug use for standardization [7].

3.4. FLUORESCENCE ANALYSIS OF POLY-HERBAL FORMULATION

The air dried plant material was subjected to fluorescence analysis under ultra violet light (254nm and 366 nm) and day light after giving treatment with various chemical and organic solvents like 1N Sodium hydroxide in methanol, 1N Sodium hydroxide in distilled water, 50% Nitric acid, 50% Sulphuric acid, 1N Hydrochloric acid [8-9].

3.5. THIN-LAYER CHROMATOGRAPHY OF POLY-HERBAL FORMULATION

Extract of poly-herbal drug in small quantity was dissolved in ethanol (analytical grade) for Thin Layer Chromatography TLC. Ready-made TLC plates (Silica gel 254 fluorescent, Merck, Germany) were used. Sample was applied on TLC plates as described by Stahl [10]. The thin-layer chromatographic technique is carried out using following two solvent systems: Ethyl

acetate: Methanol: Water (100:16.5: 13.5) and Chloroform: Methanol: Water (80: 20: 2). Iodine vapor were used for viewing spots apart from viewing under UV lamp 254 nm and 366 nm.

3.6. SCREENING OF ANTI-BACTERIAL ACTIVITY

The anti-bacterial activity of different medicinal plants five strains were explored in this study. Anti-bacterial activity of crude extract against the test organisms were determined by using agar-well method. All plates were incubated at 28 + 2°C for 24-48 hours and after incubation diameter of zone of inhibition was measured [11].

3.7. ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory activity was evaluated by the Carrageenan induced paw edema method. [12]. The results were expressed as percentage reduction in edema volume, which can be calculated by using the formula:

% of inhibition = Control-Treated/ Control x 100

3.8. FOURIER TRANSFORM INFRARED SPECTROSCOPY

Fourier Transform Infrared Spectrometry (FTIR) of DM was carried out on FT-IR model Nicolet Avator 330-FT-IR (USA) [13].

3.9. GAS CHROMATOGRAPHY-MASS SPECTROSCOPY

Gas Chromatography-Mass Spectroscopy (GC-MS) was carried out using GC-MS 1. In the GC/MS process, a sample is first injected into a gas chromatograph, where components are separated according to size and/or polarity. Then, the components pass into a device known as a mass selective detector. It's at this stage that a mass spectrum is obtained and compared against standard reference libraries in order to identify unknown components in the sample. [14].

4. RESULTS

4.1. MICROSCOPIC EVALUATION

Prominent microscopic features found were parenchymatous cells, starch grains, epicarp, mesocarp, vascular bundles, epidermis, hypodermis, endosperm, testa, schizogenous cavities, trichome, cortex and vascular bundle (See figure 1 – 7).

4.2.POLY-HERBAL POWDERED DRUG FORMULATION REACTIONS WITH CHEMICALS

Poly-herbal powdered drug was treated with different chemical reagents. See in table 1.

4.3. PHYTO-CHEMICAL IDENTIFICATION TESTS OF POLY-HERBAL FORMULATION

Poly-herbal drug was tested for the presence or absence of carbohydrates, proteins, glycosides, flavonoids, tannins, phenols and sterols. See table 2.

4.4. FLUORESCENCE ANALYSIS OF POLY-HERBAL FORMULATION

Poly-herbal drug was treated with different chemical reagents and observed under ordinary light and UV 254 nm and 366 nm. See table 3.

4.5. THIN-LAYER CHROMATOGRAPHY OF POLY-HERBAL FORMULATION

Thin-layer chromatography was carried using two different solvent systems and spots were visualized using iodine vapor and under 254 nm and 366 nm UV light. See table 4.

4.6. FOURIER TRANSFORM INFRA-RED SPECTROSCOPY

The following functional groups peaks were observed in the spectrum: 3832.46 cm-1 and 3730.31 cm-1 (OH stretch – alcohol); 2929.40 cm-1 and 2851.76 cm-1 (C-H stretch – alkanes); 2349.15 cm-1 (-C≡C- stretch alkynes); 1707.60 cm-1 (C=O aldehyde); 1601 cm-1 (C-C stretch, aromatic); 1450.17 (C-C stretch in-ring aromatic); 1315.32 (C-O, N-O stretch); 1245.86 cm-1 and 1176.39 cm-1 (stretch alcohols, carboxylic acid); 1004.77 cm-1 (C-H bend alkene); 816.80 (C-H aromatics); 686.04 (N-H wag 1°, 2° amine); 645.18 (-C≡C-H: C-H bend alkynes). See figure

The results can be seen in figure 8.

4.7. GC-MS EVALUATION

The following peaks were observed in the mass spectrum: 5.812 (2-Heptenal); 6.749 (2,4-Heptadienal); 7.540 (Limonene); 9.114 (Heptan-2-one); 9.363 (1,6-Octadien-3-ol); 10.022 (Octanoic acid); 10.652 (Cyclohexanone); 11.874 (3-Cyclohexane-1-methanol); 12.072 (Estragole); 13.214 (Propanolol); 13.742 (2-Decenal); 14.43 (Benzene, 1-methoxy-4-(1-propenyl)-); 14.525 (5,7-Dodecadiyn-1, 12-diol); 14.613 (2,4-Decadienal); 15.228 (Tricyclo[5.2.1.0(2,6)]decane, 4-methyl); 16.319 (Phenol, 2-methoxy-4-(1-propenyl)-); 16.539 (Dimethyl phthalate); 16.539 (Benzene, 1-(1,1-dimethylethyl)-2-methoxy-4-methyl-); 16.831 (Copaene); 17.966 (Caryophyllene); 18.801 (α- Caryophyllene); 19.189 (β-Elemene); 19.460 (Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-); 19.628 (Naphthalene); 21.620 (Caryophyllenyl alcohol); 21.759 (8-Isopropenyl-1,3,3,7-tetramethyl-bicyclo[5,1,0]oct-5-en-2-one); 21.935 (Caryophyllene oxide); 22.535 (12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-); 22.535 (3-Buten-2-ol,4-(2,6,6-trimethyl-2-cyclohexen-1-yl); 23.055 (Caryophyllene oxide); 23.136 (Tetracyclo [6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl); 23.590 (Caryophyllene oxide);23.897 (Isoaromadendrene epoxide); 24.944 (Methyl tetradecanoate); 25.742 (Tetradecanoic acid); 27.090 (Caryophyllene oxide); 27.807 (3,7,11,15-Tetramethyl-2-hexadecan-1-ol); 27.997 (2-Hydroxy-1,1,10-trimethyl-6,9-epidioxydecalin); 29.023 (4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol); 30.809 (Hexdecanoic acid, methyl ester); 32.478 (n-Hexadecanoic acid); 33.877 (Hexadecanoic acid, ethyl ester) ;39.932 (9,12-Octadecadienoic acid (Z,Z)-, methyl ester) ;40.357 (9,12,15-Octadecatrienoic acid, methyl ester) ;41.931 (Octadecanoic acid, methyl ester) ;42.473 (9,12-Octadecadienoic acid (Z,Z)-); 42.751 (9,12-Octadecadienoic acid (Z,Z)-); 43.337 (9,12-Octadecadienoic acid, ethyl ester);

43.571 (9,12,15-octadecatrienoate) ; 43.571 (Ethyl 9,12, 15-octadecatrienoate); 43.571 (Ethyl 9,12, octadecatrienoate); 44.501 (Octadecanoic acid, ethyl ester); 44.508 (Octadecanoic acid, ethyl ester; 45.717 (Methyl 6-cis, 9-cis, 11-trans-octadecatrienoate); 46.573 (8,11,14-Eicosatrienoic acid; 47.108 (12,15-Octadecadienoic acid, methyl ester); 47.737 (Cyclopropanebutanoic acid); 50.725 (Hexadecanoic acid, 2,3-dihydroxypropyl ester); 51.296 (1,2-Benzenedicarboxylic acid, diisooctyl ester); 53.090 (9,12-Octadecadienoic acid (Z,Z)-,2,3-dihydroxypropyl ester); 53.192 (Nonanoic acid, 9-(3-hexenylidenecyclopropylidene)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester); 53.720 (Piperine); 53.859 (Piperine); 55.521 (Piperine); 56.077 (9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol (3β,5Z,7E); 57.007 (9,10-Secocholesta 5,7,10(19)-triene-3,24,25-triol (3β,5Z,7E); 57.351 (Ethyl iso-allocholate); 57.813 (2-Butenoic acid); 58.164 (Ethyl iso-allocholate); 58.735 (1-Heptatriacotanol); 58.735 (Lup-20(29)-en-28-oic acid, 3-hydroxy-, (3β)-) ; 59.335 (Stigmasterol); 59.877 (Stigmasterol); 60.090 (β-Sitosterol); 60.631 (4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one); 60.939 (Lup-20(29)-en-3-one); 61.298 (Lupeol); 62.162 (4,4,6a,6b,8a,11,11,14b Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one); 62.887 (Lup-20(29)-en-3-ol,acetate, (3β)-). See figure 9.

4.8. ANTI-BACTERIAL ACTIVITY OF POLY-HERBAL FORMULATION

No zone of inhibition was observed as compared to standard drug. The results of anti-bacterial activity are exhibited in Table 5 below.

4.9. ANTI-INFLAMMATORY ACTIVITY

The poly-herbal extract exhibited 0.7% inhibition of paw edema. The results of anti-inflammatory activity may be observed in table 6 below.

TABLE 1: POLY-HERBAL POWDERED DRUG REACTION WITH CHEMICALS

S.No.	Treatment of powdered drug with reagents	Reactions
1	Triturated with water	Brown liquid due to dissolving of fine particles along with undissolved larger particles.
2	Shake with water	Fine particles dissolve giving the appearance of light yellowish-brown solution. Larger particles settle at bottom that disperse on shaking.
3	Pressed between filter paper	Brown color
4	Treated with 5% NaOH solution	Dark brown solution. Larger particles settle at bottom.
5	Treated with 5% NaOH solution and heated	Brownish-black solution
6	Treated with 66% H_2SO_4	Dark brown solution with insoluble larger particles

7	Treated with 5% $FeCl_3$	Brownish-yellow solution with undissolved particles settle at bottom.
8	Treated with concentrated HCl	Mud-brown color solution with undissolved particles settle at bottom.
9	Treated with concentrated H_2SO_4	Brown solution with undissolved particles settle at bottom.
10	Treated with concentrated HNO3	Yellowish-brown solution with undissolved particles settle at bottom.
11	Treated with 1N NaOH	Light brown solution with undissolved particles settle at bottom.

TABLE 2: PHYTO-CHEMICAL IDENTIFICATION TESTS OF POLY-HERBAL POWDERED DRUG

S.No.	Identification Test	Observation	Remarks
1	Wagner test	Golden-brown color clear solution	No brown/red precipitates Alkaloids: (-)
2	Hager test	Yellowish-brown solution with insoluble particles settle at bottom	No yellow precipitates. Alkaloids: (-)
3	Molish test	Light purple solution with precipitates	No violet ring junction. Carbohydrates (-)
4	Fehling test	Clear greenish-blue solution	No red precipitates. Carbohydrates (-)
5	Benedict test	Clear green solution	No orangish-red precipitates. Carbohydrates (-)
6	Modified Bontragers test	Rose pink color in the ammonical layer	Formation of rose-pink color in the ammonical layer. Anthraquinone glycosides (+)
7	Froth test	No froth formation	Absence of 1cm layer of foam. Saponin glycosides (-)
8	Foam test	No foam formation	No foam produced to persists for 10 minutes. Saponin glycosides (-)
9	Salkowski test	Golden brown/ yellow color	Triterpenes (+)
10	Libermann Burchard test	Brown ring at junction	Phytosterols (+)
11	Ferric Chloride test	Golden brown clear solution	No bluish-black color formation.

			Phenols (-)
12	Gelatin test	Light green clear solution	No white precipitates Tannins (-)
13	Alkaline reagent test	Transparent solution	Flavonoids (+)
14	Lead acetate test	Yellow precipitates	Flavonoids (+)
15	Ninhydrin test	Blue solution	Amino acid (+)
16	Xanthoprotiec test	yellow solution	Proteins (+)

TABLE 3: FLUORESCENCE ANALYSIS OF POLY-HERBAL POWDERED DRUG

S.No.	Reagent	Under Ordinary Light	Under UV Light	
			(254 nm)	(366 nm)
1	Powder + 1N NaOH in methanol	Dark brown	Brown	Black
2	Powder + Acetone	Light brown	Fluorescent yellow	Black
3	Powder + Benzene	Dark brown	Brown	Black
4	Powder + Carbon tetrachloride	Dark brown	Brown	Black
5	Powder + Chloroform	Brown	Fluorescent yellow	Black
6	Powder + Ethanol	Brown	Brown	Black
7	Powder + Ethyl acetate	Brown	Fluorescent yellow	Black
8	Powder + Methanol	Light brown	Fluorescent yellow	Black
9	Powder + Petroleum ether	Brown	Brown	Black

TABLE 4: THIN-LAYER CHROMATOGRAPHY OF POLY-HERBAL DRUG

S.No.	Solvent System	No. of spots	Rf value
1	Ethyl acetate – Methanol – Water (100: 16.5: 13.5)	1	0.78
2	Chloroform – Methanol – Water (80: 20: 2)	3	0.62, 0.69, 0.74

TABLE 5: ANTI-BACTERIAL ACTIVITY OF THE POLY-HERBAL FORMULATION

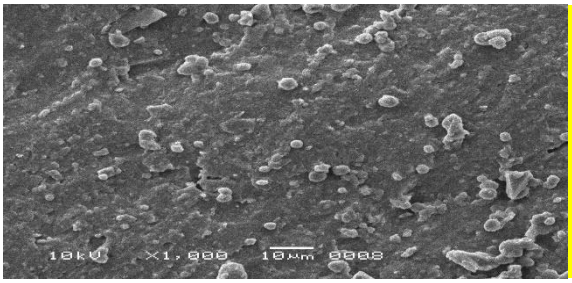
Bacteria	% Inhibition of Compound	% Inhibition of Drug
Escherichia coli	**No Inhibition**	**91.69%**
Bacillus subtilis	**No Inhibition**	**89.56%**
Staphylococcus aureus	**No Inhibition**	**94%**
Pseudomonas aeruginosa	**No Inhibition**	**88.2%**

Salmonella typhi	**No Inhibition**	**93%**

TABLE 6: ANTI-INFLAMMATORY ACTIVITY OF THE POLY-HERBAL FORMULATION

Sample Code	**Conc. (mg/ml)**	**% Inhibition**
DM	**25 µg/ml**	**0.7**

FIG 1- 7 ELECTRON POWDER MICROSCOPY OF POLY-HERBAL FORMULATION



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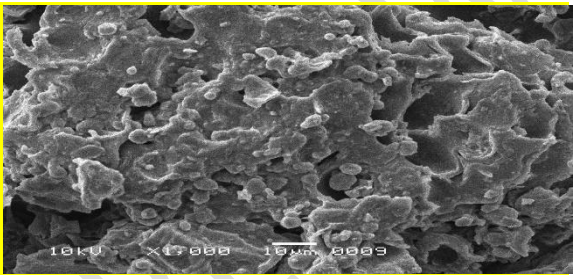

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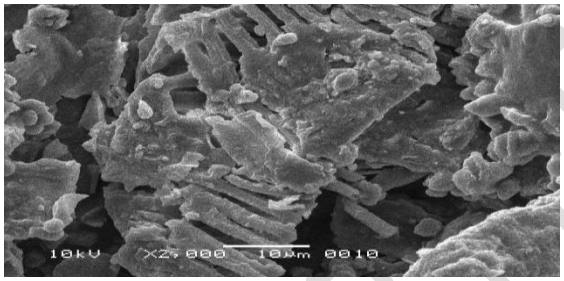

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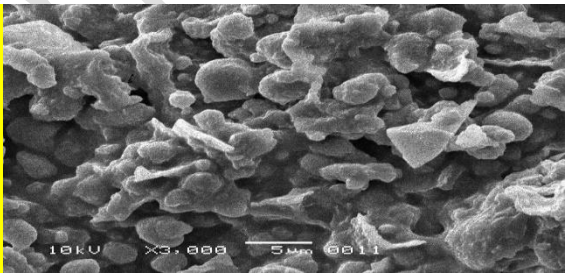

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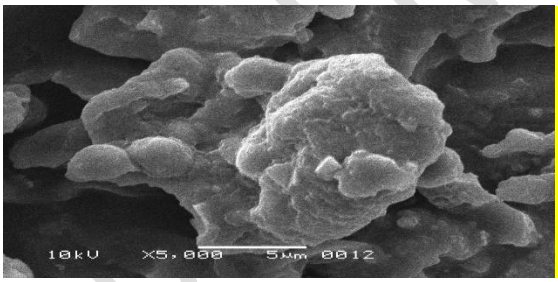

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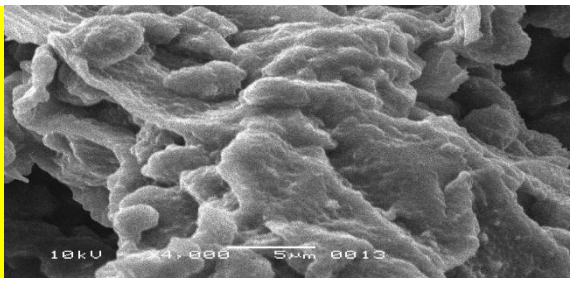

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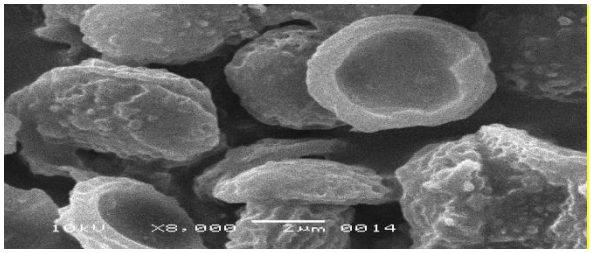

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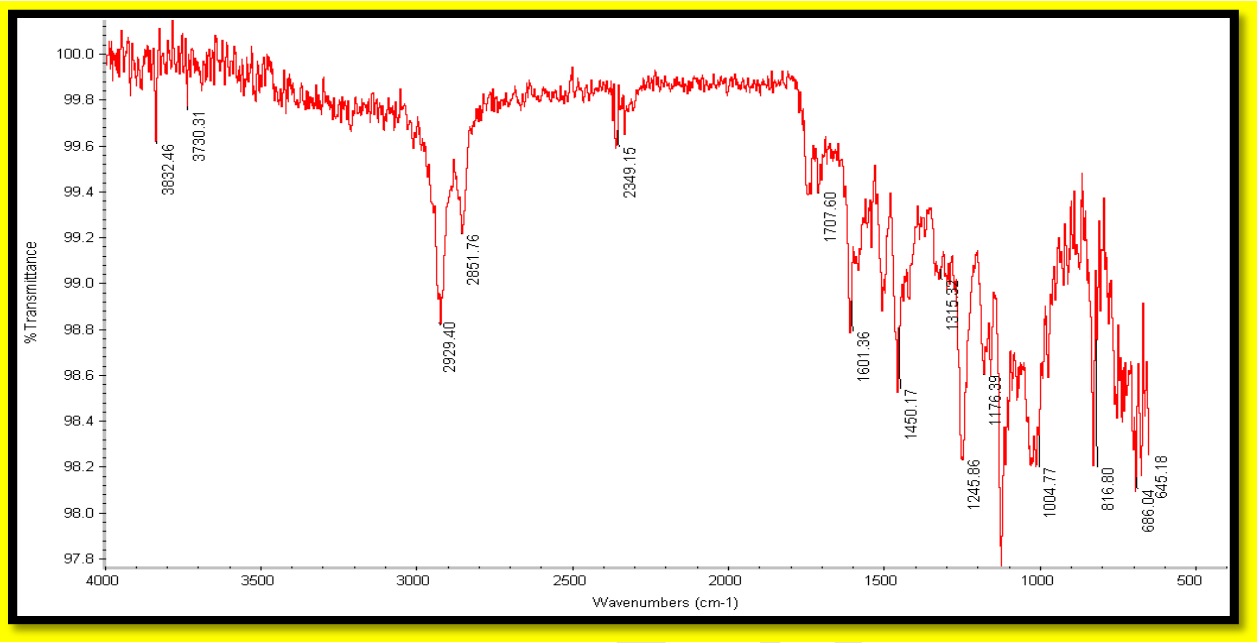

FIGURE 8: FOURIER TRANSFORM INFRA-RED SPECTRUM OF POLY-HERBAL FORMULATION

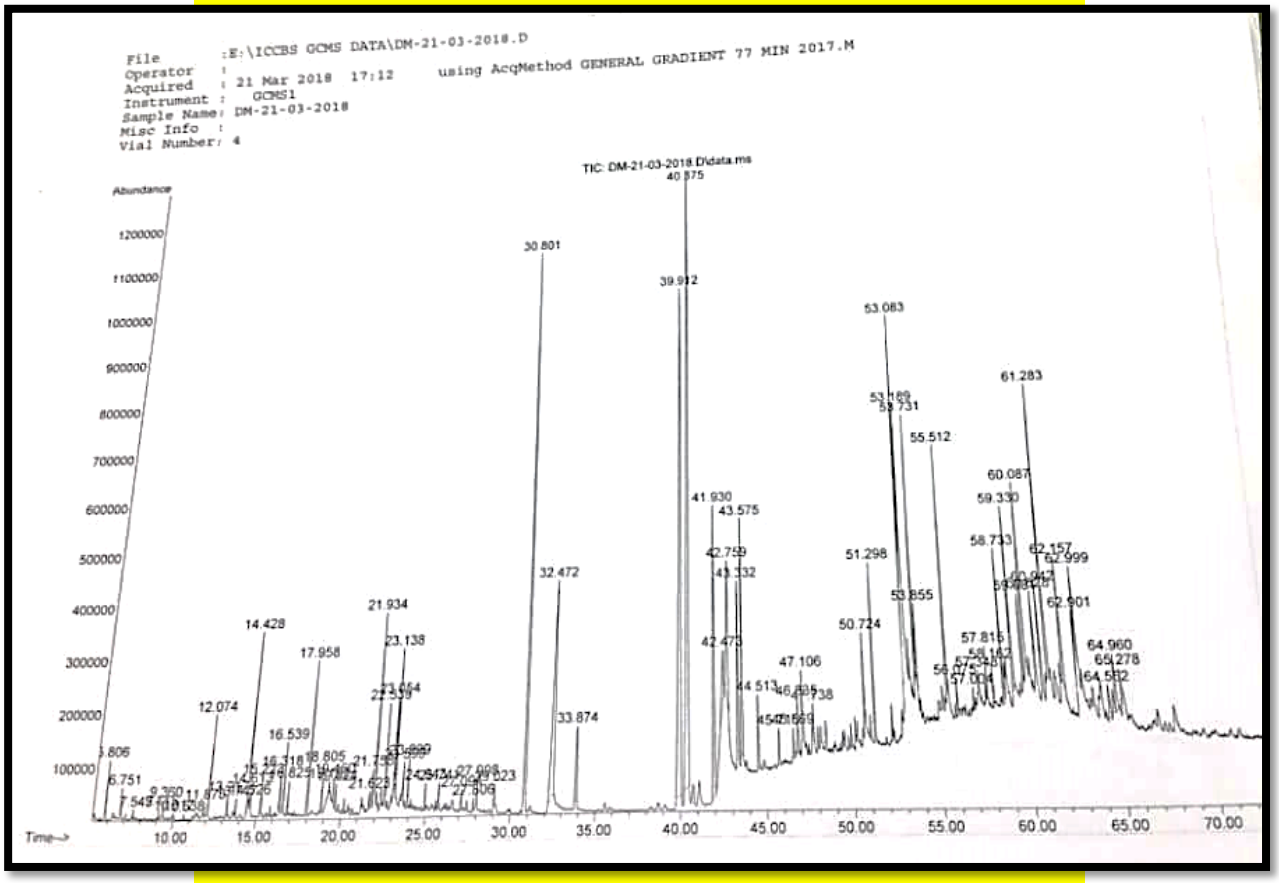

FIGURE 9: GC-MS SPECTRUM OF POLY-HERBAL FORMULATION.

5. DISCUSSION

Natural source especially the herbals are in the lime light of discovery of novel molecules with better efficacy and lesser adverse events. The researches are being carried out to explore probable mechanism of action of the pharmacologically active ingredients present in any herb or other natural source. Standardization of the herbal and other natural origin medicines is essential and plays a pivotal role in maintaining the quality control of the formulations prepared by them. In the current study the formulation was prepared with all those herbals that have already reported data of their anti-diabetic activity [15-17]. *Syzygium cumini* contains mycaminose, which possess anti-diabetic activity [18]. *Momordica charantia* contains triterpene, proteid, steroid, alkaloid, inorganic, lipid, and phenolic compounds that possess anti-diabetic activity.

Ursolic acid and chlorogenic acid, Aldose reductase inhibitors present in *Wrightia tinctoria* may be responsible for its antidiabetic potential [19]. *Gymnema sylvestre* contains triterpene saponins known as gymnemic acids, gymnemasaponins, and a polypeptide, gurmarin that may be responsible for anti-diabetic activity [20]. The results of our study revealed that the poly-herbal preparation for the treatment of diabetes mellitus contains carbohydrates, triterpenes, steroids, glycosides; polypeptides, inorganic and phenolic compounds. Due to the presence of the above mentioned constituent the formulation has potent anti-bacterial and anti-inflammatory activity.

6. CONCLUSION

The research work carried out comprehensively provides the information concerning the pharmacognostic, phytochemical, and biological studies concerning the poly-herbal formulation. Further toxicity and clinical studies are in progress to authenticate further the efficacy and safety of the drug.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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