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Evidence Based Evaluation of Pharmacological Activity and Herb-Herb interaction of Organic Extracts of Certain Natural Anti-Diabetic Mixtures

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ABSTRACT

Objective: This work aims to find a new treatment based on the development of safe natural anti-diabetic mixtures. It assesses the hypoglycemic activity of natural mixtures and determines whether there are any negative side effects from the interaction of the herbs and the herbs. **Methods**: Six natural mixtures were tested for anti-diabetic activity, which was confirmed by a pathological histological examination. We performed a GC-MS analysis on active mixtures 1 and 2, yielding 54 and 38 compounds, respectively. The major compounds were Gingerol, Butan-2-one and 4-(3-hydroxy-2-methoxyphenyl) (97 and 64.02 per cent). **Results**: Among the six samples, the organic extract of mixture 1 and 2 showed a significant reduction in BGL compared to the standard drug glimepiride at a dose of 10 mg/kg ip and demonstrated a beneficial effect on renal function in alloxan-induced diabetic mice. These results were confirmed by a histopathological study which revealed that both mixture 1 and mixture 2 had decreased morphological and ultrastructural changes in the triggered liver. Docking of cuminaldehyde, Gingerol and α-Copaenin at the active site of human pancreatic enzymes α-amylase and aldol reductase revealed that these compounds had binding affinity at the active site of the enzymes. **Conclusion**: Our results revealed the anti-diabetic activity of non-polar mixtures consisting of long chain hydrocarbons, oils and non-polar components, thus suggesting that the herbal formulation is safe and effective for the treatment and complication of type 2 diabetes.

Key words: Anti-diabetic herbs, Herb-herb interaction, Medicinal plants.

INTRODUCTION

Diabetes mellitus (DM) is a chronically high blood glucose disease that causes a major symptom of the flow of large amounts of sweet urine. The basic abnormality is insulin deficiency[1]. Recent estimates show that 382 million people worldwide have been identified as suffering from DM in 2013 and are expected to rise to 592 million by 2035, with equally increasing physiological, psychological and economic burdens on humans with life-threatening consequences[2-6]. Diabetic animal models using alloxan chemical induction could provide valuable information on the natural history of non-alcoholic fatty liver disease and improve our understanding of the mechanisms underlying this condition and its progression in diabetic patients[7]. According to previous literature reviews, cellular oxidative stress plays an important role in the progression of hyperglycemia-related tissue injury and increased reactive oxygen species output in diabetes may initiate or facilitate the development of chronic diabetic lesions in vessels, retinas, kidneys, nerves, and other organs if the diabetic organism's antioxidant defences are unable to inhibit the harmful action of those substances[8-10]. Because herbal anti-diabetic medicines were not yet commercially produced in the same quantities as modern medicines, our research focused on determining the hypoglycemic activity of natural mixtures and whether there were any negative side effects. As a result of the interaction of herbs and herbs[11]. It also hopes to find a safe combination of these herbs which are used to treat diabetes and are commercially available.

Literature review had been conducted for searching about the medicinal plants and natural products those reported to have strong antidiabetic activity and compared this study with them. We have found that more than 17 species of plants with hypoglycemic activity as a result of its richness in , alkaloids, terpenoids, flavonoids, carotenoids, and other compounds that have anti-diabetic properties. They were *Acacia Arabica*, *Allium sativum*, *Aloe vera*, *Coffea Arabica*, *Vaccinium Cyanococcus*, *Cinnamomum Zeylanicum*, *Camellia sinensis*, *Ginkgo biloba*, *Zingiberofficinale*, *Glycyrrhizaglabra*, *Linumusitatissimum*, Trigonella *foenum-graecum*, *Momordicacharantia*, *Carumcarvi*, *Coriandrumsativum*, *Curcuma longa* and *Olea europaea.*[12]. The aim of this study was to discover a healthy natural medicinal mixture with enhanced efficacy in treating diabetes.

MATERIALS AND METHODS

Design of natural mixtures

According to conducted literature review, samples of natural products that were reported to have antidiabetic activity were purchased from the local markets in Jeddah, KSA and authenticated by Dr. Samah Shabana through using The Egyptian pharmacopoeia. These samples were cleaned, shadow dried, and grinded to powder then divided to six natural mixtures were designed as shown in table 1.

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Table 1: Natural mixtures that were screened for their anti-diabetic activity.

Mixture	Natural products	Weight
Mixture 1	Blueberry (*VacciniumCyanococcus*)	50 mg
	Cinnamon (*Cinnamomumzeylanicum*)	50 mg
	Green tea (*Camellia sinensis*)	50 mg
	Ginkgo (*Ginkgo biloba*)	100 mg
Mixture 2	Green tea (*Camellia sinensis*)	150 mg
	Ginger (*Zingiber officinale*)	150 mg
	Liquorice (*Glycyrrhiza glabra*)	150 mg
	Fenugreek (*Trigonella foenum-graecum*)	150 mg
	Bitter melon (*Momordica charantia*)	150 mg
Mixture 3	Caraway (*Carum carvi*)	50 mg
	Coriander (*Coriandrum sativum*)	50 mg
	Green tea (*Camellia sinensis*)	50 mg
Mixture 4	Ginger (*Zingiber officinale*)	100 mg
	Garlic (*Allium sativum*)	100 mg
	Fenugreek (*Trigonella foenum-graecum*)	100 mg
	Turmeric (*Curcuma longa*)	100 mg
	Olives leaves (*Olea europaea*)	100 mg
Mixture 5	Fenugreek (*Trigonella foenum-graecum*)	150 mg
	Aloe (*Aloe vera*)	150 mg
	Linseed (*Linum usitatissimum*)	150 mg
	Gum acacia (*Acacia senegal*)	150 mg
Mixture 6	Liquorice (*Glycyrrhiza glabra*)	200 mg
	Caffeine	200 mg
	Ginkgo (*Ginkgo biloba*)	100 mg

Preparation of crude extracts and fractionation

Powder of each mixture was macerated in 600 ml of 70 % ethanol for about one week. The macerated mixtures were filtered, and then 50 ml of filtrate concentrated to dryness in a rotary evaporator under reduced pressure.

The remaining water residue of each extract was macerated in dichloromethane with the solvent: solute ratio of 3: 1 for 48 h with frequent shaking using separating funnel to separate aqueous extract from organic one.

Organic fractions were dried under vacuum. The dried extracts were weighted and dissolved in a definite amount of sesame oil for making dose of 10 mg/ Kg which will subjected to investigation of its anti-diabetic activity.

Investigation of antidiabetic activity of natural mixtures

Animals

54 male adult albino mice weighting 25 to 30 g were used from the animal house, Batterjee Medical College. The animals were maintained under standard environmental conditions with free access to feed and water during the experimental period in ventilated cages.

The animals were fasted for 16 hours before the experiment but allowed free access to water. All experiments were performed in the morning according to current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals. As some suffering might result from these experiments, the Batterjee medical college committee for research and ethical guidelines were followed.

The animals were kept on solid floored cages with a deep layer of sawdust to accommodate the excess of urination and cages were changed daily. All animals were euthanized by thiopental (intravenous injection, 150 mg/kg) for tissue collection.

Alloxan-induced diabetic mice

120 mg/kg of Alloxan monohydrate freshly prepared in saline then will be injected intraperitoneal to overnight fasted animals. After that, the animals were given Glucose (5 % solution) in the drinking water to prevent hypoglycemia for the next 24 hours. The non-diabetic mice will exclude from the study, and diabetes establish with non-fasting blood glucose levels of more than 200 mg/dl [13].

STUDY DESIGN

The diabetic mice (glucose >200 mg/dl) were divided into 9 groups of 6 animals each. Group I served as control received 1% gum acacia, group II served as diabetic control received 5% gum acacia, group III treated with standard glimepiride at a dose of 10 mg/kg [14]. Group IV, V, VI, VII, VIII and IX received organic extract of 6 different herbal mixtures at a dose of 10 mg/kg [15].

The freshly prepared solutions were administered daily IP for 7 days. It is better to use IP over gavage to avoid loss of quantity during the absorption phase (Table 2).

Determination of blood glucose level

Blood glucose levels were tested on the 0 day, 2st, 4th, 6th and 8st days from the start of the experiment. Blood samples were collected from the tail of the fasting animals. One millimeter of its end was cut and a drop of blood was used for blood glucose test using advanced glucometer (Roche, USA). The accuracy of glucometer was checked with O-toluidine method[16].

Biochemical analysis

Blood was collected from the retro-orbital venous plexus according to the method of kept at 37°C for 30 min, and centrifuged by 3000 RPM for 6 min. Then the separated serum was stored at -20°C for various biochemical analyses. The serum urea concentration was determined by the method for estimation of kidney function using urea diagnostic kit[17]. A liver function test including determination of albumin in serum was done using albumin diagnostic kit[18].

Histopathological studies

At the end of the experiment, the whole liver tissues from each animal were removed after cervical dislocation, and a portion of the liver was cut into two to three pieces of approximately 6 mm3 sizes and fixed in 40 percent formalin saline were dehydrated by successfully passing through different mixtures of ethyl alcohol-water, cleaned in xylene, and embedded in parafin. Thin sections of liver tissue were cut at 5 m thickness and stained with haematoxylin and eosin dye (H&E) before being mounted in neutral de-paraffinized xylene (DPX) medium for microscopic examination. The thin liver sections were mounted on permanent slides and examined under high resolution microscopy[19].

Table 2: The animals were divided into 9 groups as follow.

Group I	Negative control mice
Group II	Positive control (diabetic control mice)
Group III	Diabetic mice treated with glimepiride. The drug was given IP (10mg/kg) for 1 week
Group IV	Diabetic mice treated with organic extract of mixture 1 at dose 10 mg/kg IP for 1 week
Group V	Diabetic mice treated with organic extract of mixture 2 at dose 10 mg/kg IP for 1 week
Group VI	Diabetic mice treated with organic extract of mixture 3 at dose 10 mg/kg IP for 1 week
Group VII	Diabetic mice treated with organic extract of mixture 4 at dose 10 mg/kg IP for 1 week
Group VIII	Diabetic mice treated with organic extract of mixture 5 at dose 10 mg/kg IP for 1 week
Group IX	Diabetic mice treated with organic extract of mixture 6 at dose 10 mg/kg IP for 1 week

STATISTICAL ANALYSIS

The results of the experiments were presented as mean standard deviation. The GraphPad Prism Program was used for statistical analysis (GraphPad Software, Inc., San Diego, USA). Paired t-test was done to see any difference between the paired groups. The level of significance was set at $p < 0.05$.

GC-MS Instrument and chromatographic conditions of the active organic extracts

A Perkin Elmer Clarus 500 GC-MS (Perkin Elmer, Shelton, CT, USA) was utilized throughout the experiments. The software controller/ integrator was TurboMass version 5.4.2.1617. An Elite-1, GC capillary column, Cross bond® 100% dimethyl polysiloxane (30-meter × 0.25mm ID × 0.25 µmdf, Perkin Elmer) was used. The carrier gas was helium (purity 99.9999%) and flow rate was 0.9 mL/min. GC line temperature was 260 ˚.

Electron energy was 70 eV, and trap-emission was 100 v. The oven was programmed where the initial temperature was 90 ° (hold 4 min) to 170 ˚ (rate 10.0 ˚/min, hold 10.0 min), to 270˚ (rate 10.0 ˚/min, hold 8.0 min). Injector temperature, 265°. The injection volume was 1.0 µL, and the Split was 60%. Samples were acquired by applying the total ion chromatogram (TIC). The MS scan was from 50 to 450 m/z (500 scan/ sec). An average TIC scan of each peak at definite retention times was saved using the TurboMass software to characterize the closed peaks obtained from the MS chromatogram of the analyzed samples.

MOLECULAR DOCKING STUDIES

In silico molecular docking simulation studies of identified compounds were done for three important enzymes involved in diabetes mellitus human pancreatic α-amylase (h-PA) (PDB-4GQR), human maltase-glucoamylase (h-MGAM-C) (PDB-3TOP) and human aldol reductase (h-ALR-2) (PDB- 4QXI) by using Molecular Operating Environment (MOE®) version 2015.10[20].

Tested compounds optimization

The tested compounds Cuminaldehyde, Gingerol, and α-Copaene were created into a 3D model using builder interface of the MOE program. The structures of the tested compounds were checked by 2D depiction, formal charges on atoms and then a conformational search was conducted. All conformers were subjected to energy minimization done with MOE using the default molecular mechanic force-field mmff 94x. The database of tested compounds was then saved as MDB file for use in the molecular docking calculations.

Optimization of protein active site

The crystal structures of the h-PA, h-MGAM-C, and h-ALR-2 enzymes with their respective bounded ligands, Myricetin, Acarbose, and AK198 were obtained from the Protein Data Bank (www.pdb. org). The protein structures had been docking-ready by the addition of hydrogen atoms, as well as their standard geometry and energy minimization. MOE Alpha Site Finder searched for the active binding site in each receptor structure using all default items, and then dummy atoms were created.

Docking of the tested compounds to protein active sites

Docking of the tested compounds was performed using MOE-Dock software.To ensure a valid docking accuracy and to determine the water molecules effects, the co-crystallized ligand in each of the aforementioned enzymes was docked to its corresponding protein (in the presence and in the absence of water) and the RMSD values were calculated between the co-crystallized ligand and docked pose. The success rates obtained were excellent and the active site of the each enzyme was saved as MOE file.

The active binding site files were then loaded, and the docking tools were used. The dummy atoms as docking site, triangle matcher as placement methodology, Londond G as scoring methodology have been adjusted to their default values. Finally, the MDB files of the tested compounds were loaded, and calculations for docking were run automatically retaining 30 docking poses.

The poses obtained were studied and the poses that showed the best ligand-receptor interactions were selected and stored for calculating energy.

RESULTS AND DISCUSSION

Anti diabetic activity of natural mixtures

Blood glucose level

As compared to the normal control group, alloxan caused a substantial rise in blood glucose levels. The organic extract of mixtures 1, 2, 3 and 6 treated groups showed a substantial decrease ($P<0,05$) in blood glucose levels after one week of daily treatment compared to the diabetic control group . Herbal organic mixture extracts of 4 and 5 failed to show any hypoglycaemic activity after one week of treatment. Both of mixtures 1 and 2 showed a highest decrease in BGL as compared to the standard drug glimepiride treated group (Table 3).

Biochemical mediators

The mean values of blood urea nitrogen (BUN) CONC and blood albumin of both control and experimental groups were presented in Table 4. Alloxan-induced diabetic mice showed a significant increase ($p<0.05$) in serum urea nitrogen and decrease in serum albumin compared with the normal control. There was a significant restoration of these parameters to near normal after administration of the organic extracts of mixtures 1 and 2 more than the effect of glimepiride.

HISTOPATHOLOGY INVESTIGATION

Histopathology of the livers of mice in normal control group (figure 1) Displayed traditional hepatolobular architecture consisting of a central vein with hepatocyte radiating cords divided by sinusoids. The hepatocytes are polygonal in form, with core nuclei that are softly spotted, and conspicuous nuclei. The cytoplasm is spread regularly.

Diabetic mice treated with herbal mixture number 1 showed a protective effect on the tissue of the liver (Figure 1). Diabetic mice treated with herbal mixture number 2 displayed a marked decline in degeneration, drop in inflammation and loss of necrosis relative to diabetic control mice. Diabetic mice treated with herbal mixture number 3 showed an atrophy of the hepatocytes (A), vascular congestion (VC) and amyloidosis (AM) around the central vein in the liver tissue the same as diabetic control mice (Figure 1). Diabetic mice treated with herbal mixture number 6 showed a general Hepatocyte degeneration (HD), artery obstruction (VC), hepatocyte necrosis (NC) and penetration of the lymphocytes (Figure 1).

GC-MS analysis of the organic extracts of active mixtures (1and 2)

A variety of compounds have been identified by GC-MS examination of the organic extracts from natural mixtures 1 and 2 (figure 2). These compounds were characterized by GC-attached mass spectrometry. The numerous active components present in the dichloromethane extracts of mixtures *1 and 2* were detected by the GC-MS are shown in Table 5 and 6 respectively.

Table 3: Effect of mixtures on BGL of alloxan-induced diabetic mice.

Treatments	Blood Glucose level (mg/dl)			
	First Day	Third day	Fifth day	Seventh Day
Normal Control gp	123±11.4	112±12.56	105±11.41	109±12.21
Diabetic control gp	424±38.43*	543±54.01*	527±51.21*	576±43.21*
Glimepiride treated gp (10mg/kg)	168.25 ± 19.9[a]	225 ± 12.6[a]	117.7 ± 14.19[a]	187.7 ± 12.1[a]
Mix 1 treated gp (10mg/kg)	257 ± 24.2[a]	208 ± 19.3[a]	170 ± 14.3[a]	166 ± 20.9[a]
Mix 2 treated gp (10mg/kg)	234 ± 73.2[a]	223 ± 25.5[a]	271 ± 23.9[a]	157 ± 32.45[a]
Mix 3 treated gp (10mg/kg)	233 ± 12.8[a]	315 ± 21.6[a]	346 ± 19.7[a]	371.5 ± 22.8[a]
Mix 4 treated gp (10mg/kg)	366 ± 24.6[a]	423 ± 45.5[a]	586 ± 45.3	491 ± 50.9
Mix 5 treated gp (10mg/kg)	397 ± 29.1	287 ± 29.18[a]	467 ± 33.3	600 ± 54.5
Mix 6 treated gp (10mg/kg)	522 ± 54.3	302 ± 22.1[a]	291 ± 39.5[a]	194±22.2[a]

*P < 0.05 compared with normal control mice, [a]P < 0.05 compared with diabetic control mice

Table 4: Effects of daily administration of the organic extracts of herbal mixtures on serum level of urea and albumin in alloxan-induced diabetic mice for 1 week.

Treatments	Biochemical	
Groups	Mean	
	Urea (g/dl)	Albumin (g/dl)
Group1: Normal Control	30.27±0.34	3.11±0.47
Group2: Diabetic control	47.71±7.92*	2.11±0.33*
Group3: Glimepiride treated	35.05±0.27a	2.17±0.6 a
Group4: Mixture 1 treated	32.21±0.01a	2.85±0.01a
Group5: Mixture 2 treated	27.95±1.83a	3.97±0.48*
Group6: Mixture 3 treated	35.46±0.01a	0.99±0.01a*
Group7: Mixture 5 treated	41.416±2.33*	3.75±0.19*
Group8: Mixture 6 treated	43.58±4.43*	3.59±0.5*

* Significant different from normal control group,
[a] Significant different from diabetic control group.

Table 5: Normalized area percentage of GC-MS characterized compounds of dichloromethane extract of MIX1.

Rt. Min	Name	m/z	area %	Rt. min	Name	m/z	area %
3.087	2(z)-Heptenal	112	2.65	8.908	Parabanic acid	128	0.41
3.147	Benzaldehyde	106	1.03	9.399	4,4,6-Trimethyl-cyclohex-2-en-1-ol	140	1.60
3.457	Caproic acid hexanoic)	116	0.48	9.975	2-Undecenal	168	0.61
3.773	2.2-Amylfuran	138	1.36	10.160	N-Capric acid	172	0.42
3.853	Caprylic aldehyde (Octanal)	128	0.15	10.190	Vanillin	152	0.10
3.953	6-Azabicyclo[3.2.1]octane	111	0.14	10.496	Ethyl caprate	200	0.67
4.028	2.4-Hydroxy-2-hexenoic acid lactone	112	0.08	10.676	Cumarin	146	1.14
4.434	3-Octen-2-one	126	0.47	10.786	*trans*-Cinnamic acid	148	0.96
4.785	2-Octenal	126	1.66	11.673	Azelaic acid monoethyl ester	200	5.33
5.055	1-Chlorooctane	148	0.62	11.944	4-Hydroxy-2-methoxycinnamaldehyde	162	0.96
5.176	Pentylcyclopropane	112	0.29	12.219	L-calamenene	202	0.49
5.266	Enanthylic acid	130	0.13	12.730	Lauric acid (Dodecanoic)	200	2.59
5.727	Pelargonaldehyde	142	1.81	13.116	Dodecanoic acid, ethyl ester	228	2.75
5.932	Trans-2-heptenoic acid	128	0.12	13.291	1-Cetene	224	10.59
6.704	2-Nonenal	140	0.38	13.361	Benzophenone	182	1.11
6.949	Dimethylparabanic acid	142	1.11	13.872	Methyl 10-oxo-8-decenoate	198	2.67
7.139	Caprylic acid	144	1.00	14.724	Paramethadione	157	0.45
7.465	Ethyl caprylate	172	0.67	16.617	Myristic acid	228	7.55
7.605	2,4-Nonadienal	138	0.88	17.409	Ethyl myristate	256	9.44
7.771	2-Ethyl-3-methylmaleimide	139	0.34	19.077	2-Pentadecanone, 6,10,14-trimethyl	268	1.93
7.891	2-Sec-Butylcyclohexanone	154	0.55	20.886	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	276	0.96
7.951	2-Anisaldehyde	136	0.24	24.217	Palmitic acid	256	56.13
8.056	Cuminaldehyde	148	0.47	24.708	Ethyl palmitate	284	100.00
8.226	2-Decenal	154	0.96	26.361	n-Heptadecanol-1	256	4.85
8.317	2-Decen-1-ol	156	0.36	27.603	Steric acid	284	2.73
8.372	Cinnamaldehyde	132	6.59	27.984	Stearic acid, ethyl ester	312	14.53
8.442	2-Decenal	154	7.35	31.887	.Ethyl docosanoate	368	1.00

* Percentage of matching given by NIST2008 database.

Table 6: Normalized area percentage of GC-MS characterized compounds of dichloromethane extract of MIX2.

Rt, min	Name	m/z	area %	Rt, min	Name	m/z	area %
2.956	2-Heptenal, (E)-	112	0.62	11.703	Curcumin	202	16.69
3.087	2-Heptenal, (Z)-	112	15.62	11.954	(+)-β-Selinene	204	3.92
3.187	1,6-Octadiene, 3,7-dimethyl-, (S)-	138	0.42	12.024	γ-Murolene	204	9.57
3.402	Caproic acid	116	1.24	12.089	(+,-)-β-Bisabolene	204	2.98
3.507	1-Octen-3-ol	128	0.66	12.264	β-Sesquiphellandrene	204	8.12
3.638	2,4-Heptadienal, (E,E)	110	1.33	12.470	coniferyl alcohol	180	1.59
3.813	Caprylic aldehydes	128	8.74	12.545	Hedycaryol	222	1.89
6.027	Benzene, 1-methyl-4-(1-methylpropyl)	148	2.99	13.336	Lanceol, cis	220	2.76
7.004	Borneol	154	2.31	13.542	Butan-2-one, 4-(3-hydroxy-2-methoxyphenyl)-	194	64.02
7.390	α-Terpinol	154	1.16	13.647	Cyclopentanone, 3,3,4-trimethyl-4-(4-methylphenyl)-	216	11.39
7.565	Caprinaldehyde	156	25.35	13.762	Lanceol, cis	220	5.56
8.432	2-Decenal, €	154	3.17	14.063	Benzenebutanal, γ,4-dimethyl-	176	14.39
8.923	2,4-Decadienal	152	6.60	14.128	β-Selinenol	222	2.62
8.988	2-Undecanone	170	1.37	14.729	Lanceol, cis	220	4.11
9.243	2,4-Decadienal	152	8.03	15.029	Bergamotol, Z-α-trans-	220	7.19
9.865	Carbamic acid, N-phenyl-, 1-methyl-1-(4-methylcyclohex-3-enyl)ethyl ester	273	1.30	28.690	Gingerol (81%)*	294	100.00
10.120	N-Capric acid	172	1.29	29.692	Gingerol (97 %)*	294	59.50
10.401	Benzenebutanal, γ,4-dimethyl-	176	1.45	30.815	Gingerol (59 %)*	294	17.45
10.476	α-Copaene	204	0.52	31.040	2-(3,7-Dimethyl-octa-2,6-dienyl)-4-methoxy-phenol	260	5.48

* Percentage of matching given by NIST2008 database.

Table 7: Energy docking ratings (kcal / mol) obtained from the MOE for compounds Cuminaldehyde, Gingerol, and α-copaenewith the corresponding ligands in h-PA, h-MGAM-C and h-ALR-2 enzymes.

Human pancreatic α-amylase (h-PA)							
Comp. No.	**Score**	**rmsd_refine**	**E_conf**	**E_place**	**E_score1**	**E_refine**	**E_score2**
Cuminaldehyde	-4.4331	1.8322	6.8487	-28.0686	-8.2481	-17.8222	-4.4331
Gingerol	-6.2050	1.4665	-21.4018	-53.3649	-12.1882	-26.9625	-6.2050
α-copaene	-4.9636	1.1082	52.3987	-18.9712	-7.7147	-11.0097	-4.9636
Myricetin	-5.5827	1.3889	-4.9237	-69.1133	-18.6686	-24.8616	-5.5827
Human maltase-glucoamylase (h-MGAM-C)							
Cuminaldehyde	-5.0865	2.4575	6.4266	-32.3048	-7.1173	-21.3311	-5.0865
Gingerol	-6.5708	1.6988	-19.7463	-52.6272	-10.6130	-31.2656	-6.5708
α-copaene	-4.5777	4.1419	45.1840	-38.2187	-7.6583	-17.0778	-4.5777
Acarbose	-9.6234	1.5844	269.4543	-37.6630	-11.3820	-54.9423	-9.6234
Human aldol reductase (h-ALR-2)							
Cuminaldehyde	-5.1308	1.2869	6.5917	-37.0623	-8.0148	-20.4488	-5.130
Gingerol	-7.8558	1.4574	-18.3303	-83.6102	-13.8423	-39.7450	-7.8558
α-copaene	-5.3897	1.6761	52.3228	-25.6032	-9.1911	-12.6999	-5.3897
AK198	-8.0848	2.3050	-51.3488	-68.9124	-11.3111	-46.1859	-8.0848

Molecular docking of selected compounds

Molecular docking is considered as an important tool to study the interactions between certain ligands and the binding site of the corresponding protein. In this study, to explore the molecular targets that might be involved in the herbal mixtures 1 and 2 antidiabetic mode of action, **Cuminaldehyde**, **Gingerol**, and **α-copaene**, which represent the antidiabetic components in the mixtures, were docked into the active binding sites of **h-PA**, **h-MGAM-C** and **h-ALR-2** enzymes which are involved in the diabetes mellitus pathogenesis and complications[21-23].

The results were illustrated in (Table 7 and Figure 6). For **h-PA** enzyme that mediated starch digestion to produce maltose, maltotriose, and some other alpha 1, 4 and 1,6- linked oligo-glucans.

The binding free energy of the tested compounds exhibit favorable docked complexes with the active site of target protein with significant docking scores of -4.4331, -6.2050, and -4.9636 kcal/mol for **Cuminaldehyde**, **Gingerol**, and **α-copaene**, respectively, compared with **Myricetin** docking score (-5.5827 kcal/mol) (Table 7).

Regarding the interactions at **h-PA** enzyme binding site, while, Cuminaldehyde showed binding interactions with HIS-101and ASP-197, Gingerol and α-copaene had binding interactions with TRP-59 compared to Myricetin that showed binding interactions with TRP-59 and ASP-300 (Figure 3-I).

On the other hand, for **h-MGAM-C** enzyme that is involved in the production of glucose units by hydrolyzing different oligoglucans. The binding free energy of the tested compounds showed docked complexes with the active site of the target protein with docking scores

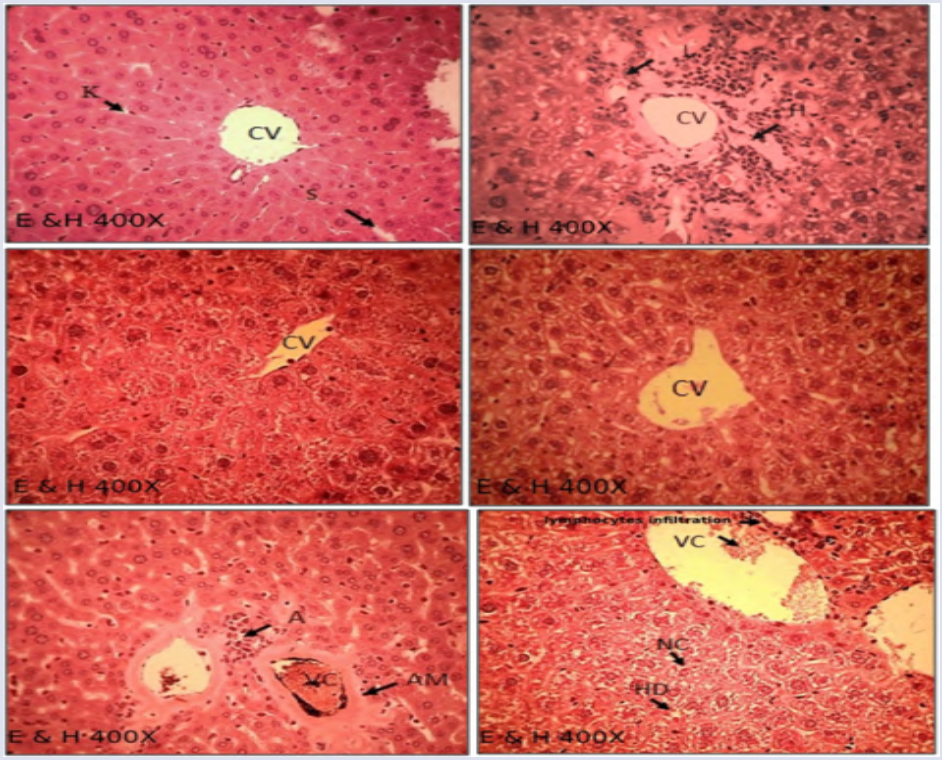

Figure 1A. A portion of normal mouse liver that displays natural liver architecture: central vein (CV), hepatocytes arranged in cord shape. Cords are divided from Kupffer cells (K) by the sinusoids (S). **Figure 1B.** A section of liver from diabetic control mouse displaying invasion of the lymphocytes (L), hepatocyte degeneration (H) along central vein (CV). **Figure 1C.** A section of liver from diabetic mouse treated with herbal mixture number 1 showing decrease in congestion and absence in necrosis. **Figure 1D.** A sectional representative of liver from diabetic mouse treated with herbal mixture number 2 displaying reduced degeneration, reduced inflammation, and absence of liver necrosis. **Figure 1E.** A section of liver from diabetic mouse treated with herbal mixture number 3 showed an atrophy of the hepatocytes (A), vascular congestion (VC) and amyloidosis (AM) around the central vein in the liver tissue. **Figure 1F.** A portion of liver from diabetic mouse treated with herbal mixture number 6 demonstrating hepatocyte degeneration (HD), vascular obstruction (VC), hepatic necrosis (NC), and invasion of lymphocytes.

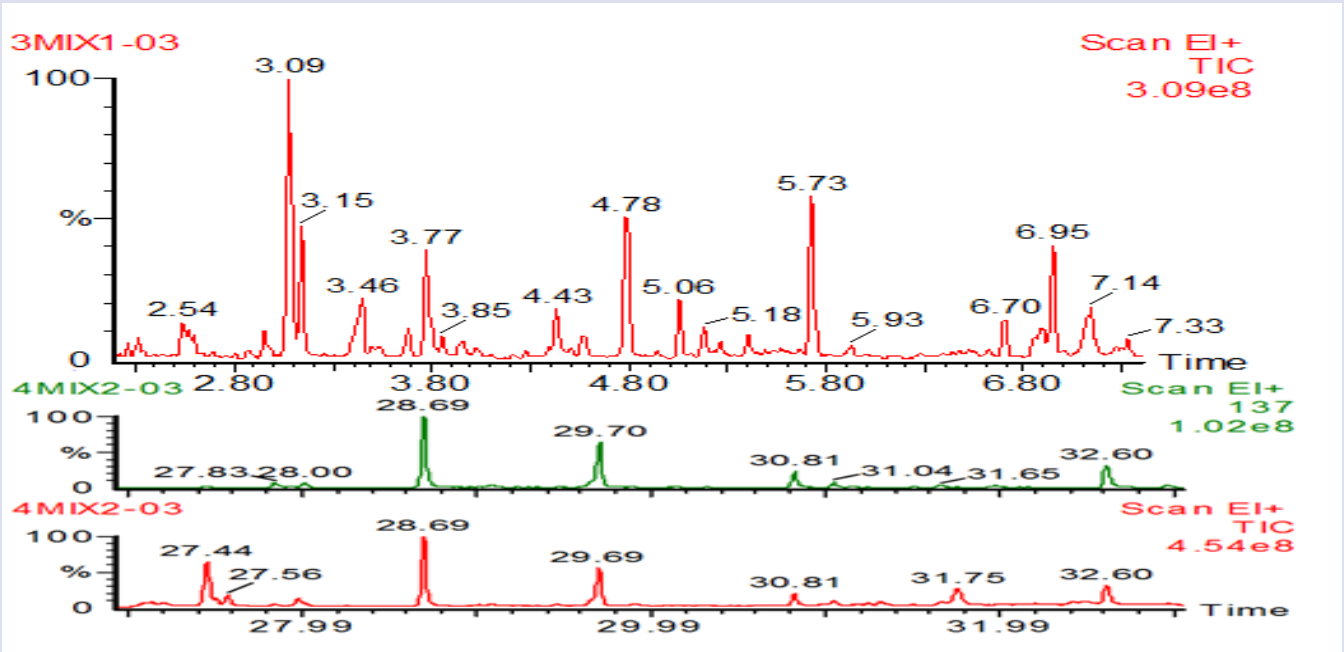

Figure 2: GC-MS of the extracts from natural mixtures 1 and 2.

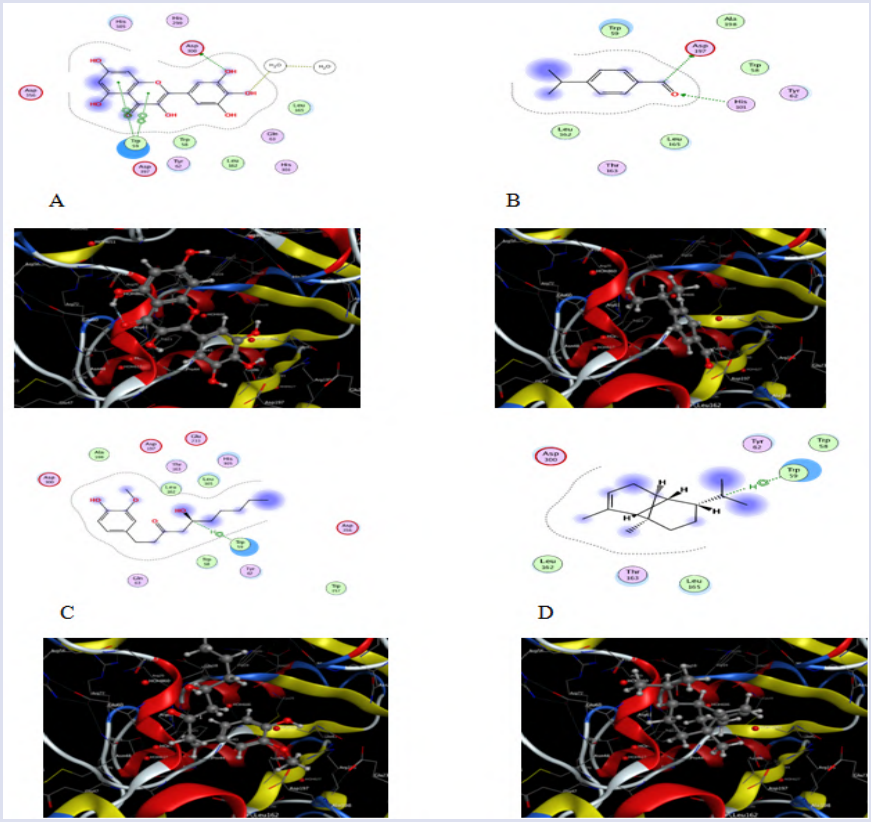

Figure 3 (1): Docking of compounds (A) Myricetin, (B) Cuminaldehyde, (C) Gingerol and (D) α-copaenein to h-PA active sites.

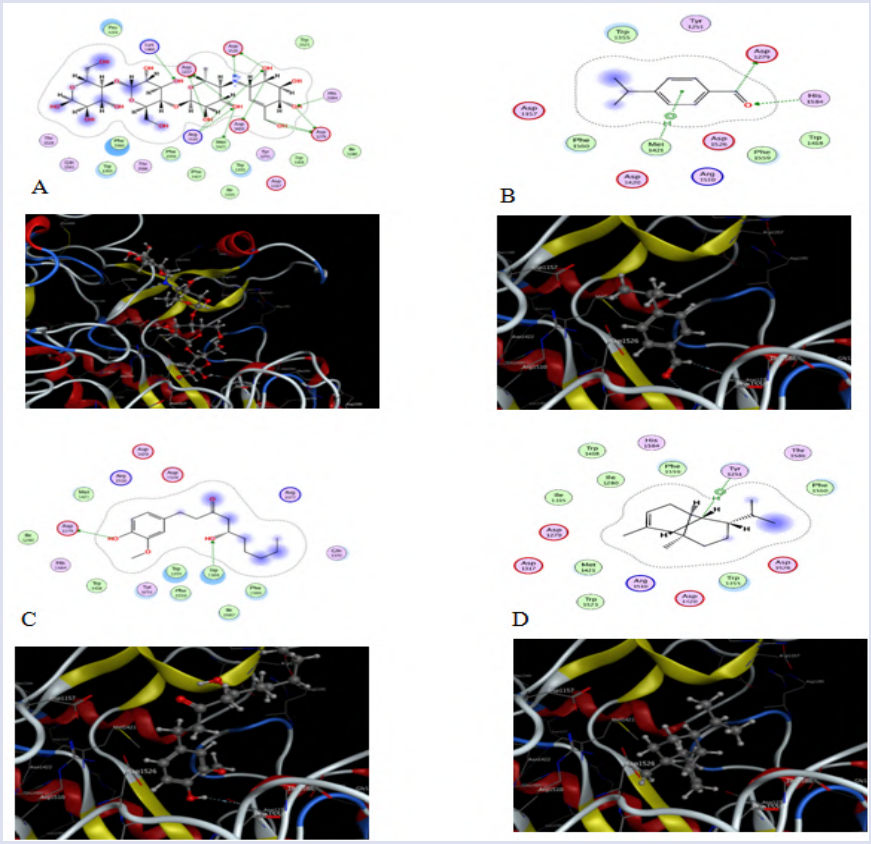

Figure 3 (II): Docking of compounds (A) Acarbose, (B)Cuminaldehyde, (C) Gingerol and (D) α-copaenein to h-MGAM-C active sites.

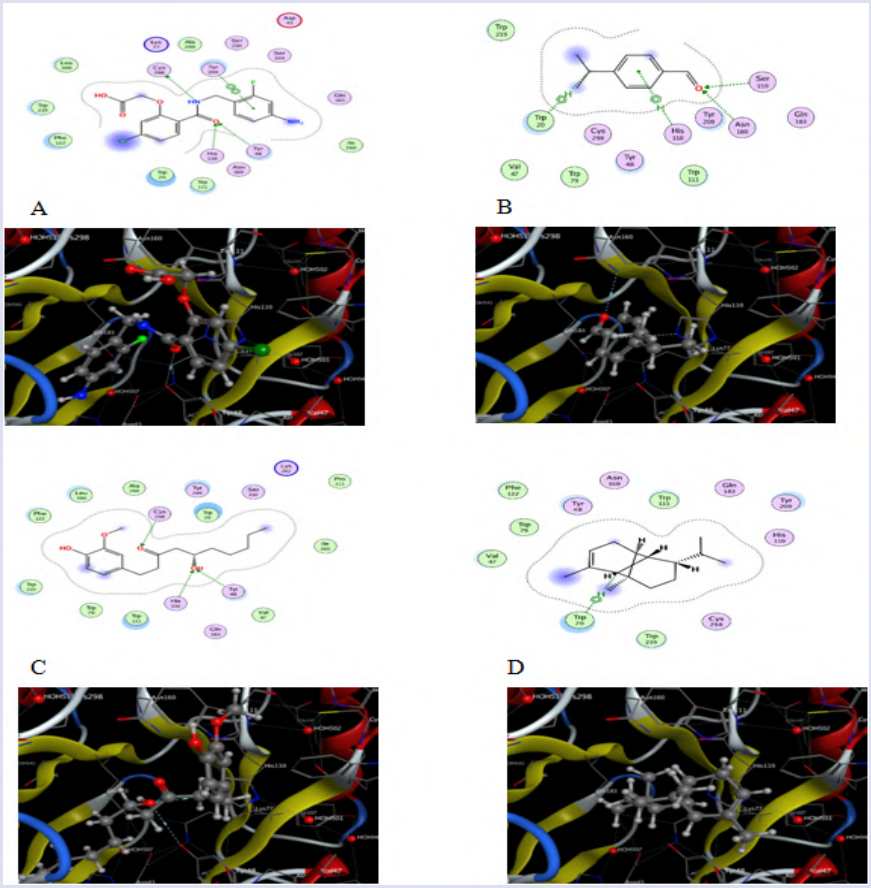

Figure 3 (III). Docking of compounds (A) AK198, (B) Cuminaldehyde, (C) Gingerol and (D) α-copaenein to h-ALR-2 active site.

of -5.0865, -6.5708, and -4.5777 kcal/mol for Cuminaldehyde, Gingerol, and α-copaene, respectively, compared with Acarbose docking score (-9.6234 kcal/mol) (Table 7).

Regarding **h-MGAM-C** ligands interactions at enzyme binding site, the antidiabetic **Acarbose** was found to afford several interactions with amino acids ASP-1157, ASP-1279, ASP-1420, MET-1421, LYS-1460, ARG-1510, ASP-1526, and HIS-1584. The results showed that the tested compounds exhibited interactions with some of aforementioned amino acids at enzyme binding site, such as Cuminaldehyde showed binding interactions with ASP-1279, MET-1421, and HIS-1584, Gingerol had binding interactions with ASP-1279, and TRP-1369 as will as α-copaene had binding interactions with TYR-1251 (Figure 3-II).

For the third target enzyme, **h-ALR-2** that is involved in glucose reduction and accumulation of sorbitol, the main causative of diabetes complications and the binding free energy of the tested compounds showed favorable docked complexes with the active site with significant docking scores of -5.1308, -7.8558, and -5.3897 kcal/mol for Cuminaldehyde, Gingerol, and α-copaene, respectively. Compared with **AK198** docking score (-8.0848 kcal/mol) (Table 7).

Regarding the interactions at **h-ALR-2** enzyme binding site, **AK198** showed binding interactions with TYR-48, HIS-110, TYR-209, and CYS-298 amino acids. while, **Gingerol** showed excellent binding interactions similarity with **AK198** as it bound with three out of four amino acids that interacted with **AK198** (TYR-48, HIS-110, and CYS-298 amino acids)[24]. While, Cuminaldehyde interacted with amino acids TRP-20, HIS-110, SER-159, and ASN-160, α-copaene had binding interactions with only TRP-20 amino acid (Figure 3-III).

From the results among the three tested compounds, Gingerol, had the best binding free energy and showed the best binding interactions with all target enzymes.

Score; lower scores are more favorable, **rmsd_refine**; The mean square root deviation of the pose from the docking pose relative to the position of the co-cristal ligand, **E_conf**; The conformer free binding energy, **E_place**; free binding energy from the stage of placement, **E_score 1**; free binding energy from stage one of the rescoring, **E_refine**; free binding energy from the refinement stage, **E_score 2**; free binding energy from the second rescoring stage.

Treatment employing two or more herbs in combination known as, "polyherbal therapy" has the benefit of achieving optimum medicinal effectiveness at a lower dosage than a conventional herbal remedy. Because of the existence of large spectrum of phyto-bioactive ingredients, polyherbal therapy may have synergistic, potential pharmacological properties inside itself.

The present study focused on evaluating the pharmacological activity and possible benefit associated with the combination therapy relative to traditional anti-diabetic treatment, Glimepiride. Hyperglycaemia caused by alloxan has been identified as a valuable laboratory model for testing the action of hypoglycemic agents because alloxane, β-cytototoxin induces significant degradation of β-cells in Langerhans islets which result in decreased synthesis and release of insulin.

Diabetes caused by alloxane is characterised by a reduction of body weight and elevated food consumption. Body weight loss may result from protein wastage due to carbohydrate metabolism deficiency and excessive tissue protein breakdown[25]. The decrease in blood glucose

was more significant (P<0.05) with the combination therapy than the single treatment. There appears to be a common assumption that the synergistic therapeutic effects of these mixtures were resulting from the interactions between the numerous bioactive constituents within the herbal preparations.

Our results showed that alloxan caused significant increase in serum urea and decrease in serum albumin level in diabetic animals when compared with normal control mice. These results are agreed with those reported by previous report[26]. This may due to metabolic disturbance in diabetes reflected in high activities of xanthine oxidase, lipid peroxidation, and increased triacylglycerol and cholesterol levels. Similar results were showing the increased concentrations of urea and creatinine due to excessive lipolysis in severe diabetes mellitus leading to ketosis and later on to acidosis[27]. Alloxan-induced diabetes triggered liver morphological and ultrastructural changes that closely resembled human disease, ranging from steatosis to steatohepatitis and liver fibrosis, therefore the current study focused on liver histopathology study[28].

Treatment of diabetic mice with the herbal mixtures 1 and 2 or glimepiride reduced fasting blood glucose level, serum urea nitrogen comparing to diabetic control group. Such an effect may be accounted for in part by a decrease in the mice of intestinal glucose absorption achieved by an extra pancreatic action including the stimulation of peripheral glucose utilization or enhancing glycolytic and glycogenic process with concomitant decrease in glycogenolysis and gluconeogenesis[29].

Many Studies showed that the rate of kidney cell damage (nephropathy) in diabetes disorders increased significantly hyperglycaemia increases the production of free radicals by auto-oxidation of glucose and the increase of free radicals can cause damage to the renal cells[30]. Reduction in plasma albumin was observed in alloxan induced mice which may be due to microproteinuria and albuminuria, which is an important clinical marker of diabetic nephropathy or may be due to increased protein catabolism[30, 31].

In plant phytochemical screening, the presence of flavonoids, alkaloids, glycosides, phenolics and tannins is likely responsible for the antidiabetic effects and enhancement of kidney and liver functions[32]. For this reason, we concentrated on organic extract for discovering the activity of long chain hydrocarbons, oils and non-polar components. It could be inferred from the overall results of the biochemical and histopathological tests that the organic extract from mixtures 1 and 2 showed a beneficial effect on the renal function in alloxan-induced diabetic mice (especially at a dose of 10 mg / kg IP).

Both of the organic extracts of mixture 1 and 2 showed a protective effect on the liver tissue in addition to its potent anti-diabetic activity. According to GC-MS results the major components in mixture 1 were palmitic acid, ethyl palmitate, in addition to minor amount of cuminaldehyde. It was reported that cuminaldehyde showed reduction in blood glucose level without causing hypoglycemia or β-cell exhaustion[33]. This fact was confirmed by our histopathological results[34].

It was previously mentioned that exposure to diet with high amount of palmitic acid inhibits the autophagic flux and decreases insulin sensitivity in hypothalamic neurons. Also palmitic acid showed lipotoxicity through elevation of triglyceride and oxidative stress[35].

Our finding demonstrate that the potent antidiabetic activity of herbal mixture 1 was related to cuminaldehyde despite its lower concentration in addition to its protective effect against the lipotoxicity and action of palmitic acid on liver tissue. All these results together can introduce strong evidence on interaction between bioactive compounds in herbal mixtures. For mixture 2 the major compounds were Gingerol, Curcumin, several hydrocarbons and minor amount of α-Copaene.

It was reported that Gingerol has antidiabetic activity through various mechanisms, curcumin also decrease blood glucose level, and the levels of glycosylated hemoglobin in diabetic rats over the ruling of polyol pathway[36, 37]. α-Copaene as one of the essential oil components of *Sabina chinensis* had an inhibitory activity of α-amylas[38].

Our results revealed that herbal mixture 2 with several antidiabetic ingredients can have multiple targets, suggesting that activity can be accomplished by the synergistic and dynamic interactions between these multiple components.

Molecular docking of cuminaldehyde, Gingerol and α-Copaenin at the active site of the human pancreatic enzymes α-amylase, maltase-glucoamylase and aldol reductase showed that all these test compounds had a strong binding affinity at the active sites of the enzymes. Implying that these substances could be mixed with promising drugs in the future for the treatment of diabetes.

Histopathological results revealed that the synergistic action between these several potent antioxidant materials could decrease the progress of liver injury.

The organic extracts of herbal mixture number 3, 4, 5 and 6 failed to show any protection in the progress of liver injury suggesting that the reason is inhibitory action between their components despite anti-diabetic activity of each component extract alone.

Both of the mixtures 3 and 6 showed a potent harmful effect on the liver tissues as compared to diabetic control mice which give strong evidence for herb- herb interaction.

CONCLUSION

The current study found that the combination therapy, as designed in mixtures 1 and 2, provided medical benefits. As a result, a safe and effective herbal mixture for the treatment of diabetes was created. This mixture contains anti-diabetic Gingerol compounds that work to lower glucose levels, as well as Palmitic acids, Cuminaldehyde and α-copaene which improve hyperglycemia. A potent and safe anti-diabetic herbal formula will be suggested.

The herb-herb interaction was proven in this study through synergistic action of compounds in mixture 1and 2 and inhibitory action of compounds in mixtures 4 and 5 in addition to harmful interaction between compounds in mixtures 3 and 6.

ABBREVIATIONS

Diabetes mellitus (DM); *De-paraffinized xylene (DPX);* Gas chromatography mass spectromery (GC-MS); Total ion chromatogram (TIC); Human pancreatic α-amylase (h-PA); Human maltase-glucoamylase (h-MGAM-C); Human aldol reductase(h-ALR-2); Molecular Operating Environment program (MOE); vascular congestion (VC); Amyloidosis(AM); Hepatocyte degeneration (HD); Hepatocyte necrosis(NC); Central vein(CV); The conformer free binding energy (E_conf); Free binding energy from the stage of placement (E_place); Free binding energy From stage one of the rescoring (E_score 1); Free binding energy from the refinement stage(E_refine); Free binding energy from the second rescoring stage (E_score 2).

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AVAILABILITY OF DATA AND MATERIALS

The data sets from this analysis are available upon request from the corresponding author.

AUTHORS' CONTRIBUTIONS

Conceptualization Asmaa Sayed Abd Elkarim, Samah Shabana, A.H. A.and A.M. Elgamal. Data curation, GC Mass A. Khedr. Pharmacology, Histopathology, Biochemistry M. F. Shalaby and Roula Bayram, Molecular docking, Radwan Elhaggar. Resources A. S. Abd Elkarim and R. Bayram.Writing original draft S. Shabana.Writingreview and editing A. S. Abd Elkarim. InvestigationS. Shabana and A. S. Abd Elkarim. Formal analysis Radwan Elhaggar, A. Khedr and M. F. Shalaby.

COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

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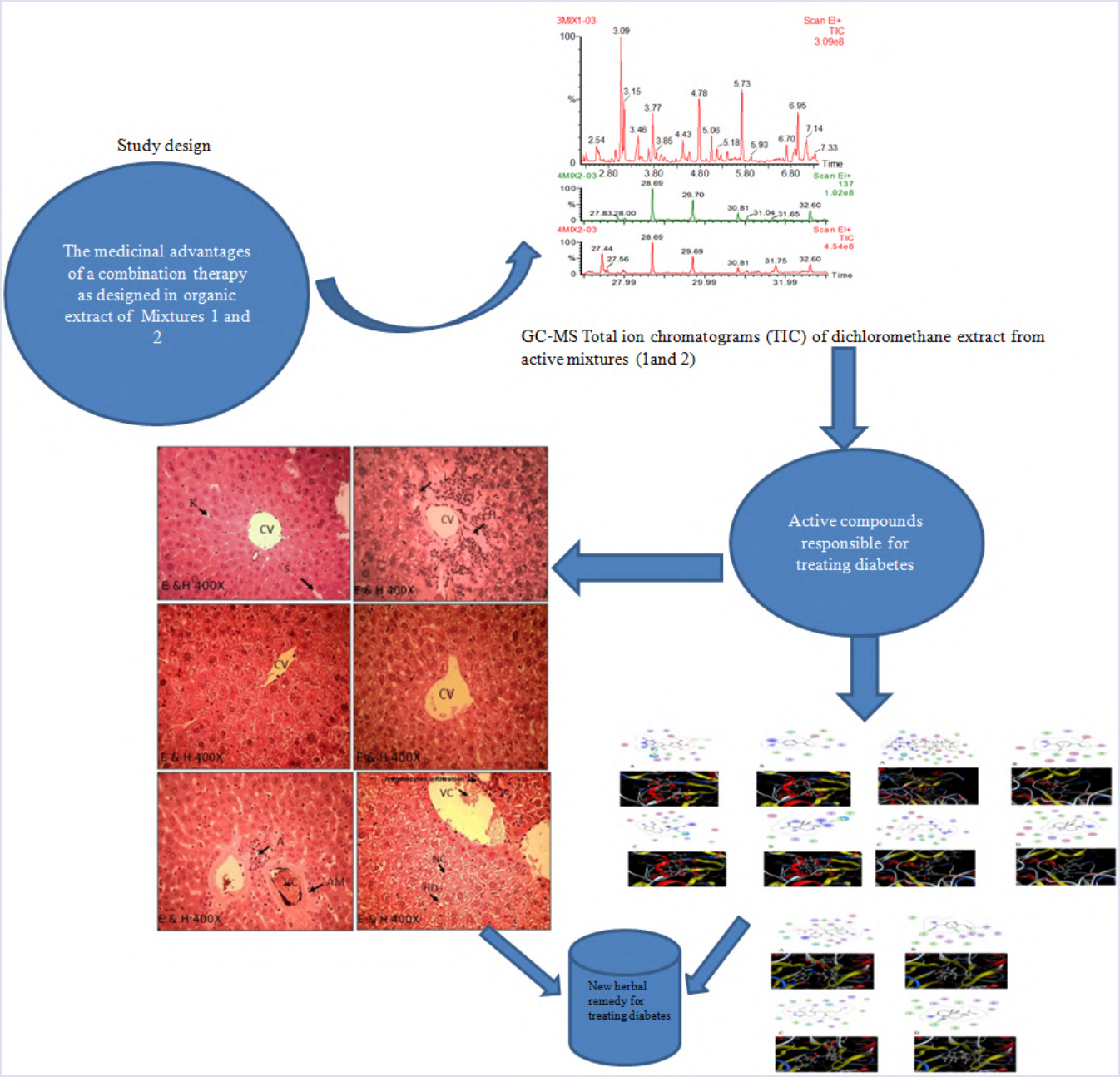
GRAPHICAL ABSTRACT



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