Research article

Hypoglycemic potential of combined methanolic extract of *Nigela sativa* (black cumin) and *Cicer arietinum* (chickpea)

Zoha Malik[1], Muhammad Tahir Akhtar[1], Abu Bakar Siddique[1], Nargis Sultana[1], Muhammad Imran Irfan[1], Rahman Qadir[1], Suleman Saleem[1], Syed Naseer Hussain Shah[1] and Mubshara Saadia[2*].

[1]Institute of chemistry, University of Sargodha, 40100, Punjab, Pakistan.

[2]Department of chemistry, Ghazi University, 32200, Punjab, Pakistan.

* Correspondence: msadia@gudgk.edu.pk; Tel.: +923347500984

Abstract: In spite of the rapid progress in the field of medicine, humanity still striving for cure of diabetes, a disorder of metabolism. From literature study it is inferred that no one therapeutic agent is isolated and/ or synthesized to treat diabetes, but to manage the disease. Therefore, it is of much importance to address this medical issue with phytochemical synergism for boosting immunity and limiting the severe side effects of synthetic hypoglycemic agents. The antioxidant and hypoglycemic effects of black seeds and chickpea have been determined previously, however the combined hypoglycemic effect of these seeds has not been studied until now. In present study the combined methanolic extract (CME) of *Nigella sativa* (black cumen) and *Cicer arietinum* (chickpea) seeds has shown the significant antioxidant potential as revealed by its significantly reduced IC50 value for radical scavenging activity in DPPH assay (-36.39%, $p<0.001$) compared to the standard compound butylated hydroxyanisole (BHA), and also due to the presence of significant ($p<0.001$) amount of total phenolic contents (TPC) in the mixture (254.2±2.75 mg GAE/g of dry weight). The CME was also fractionated through column chromatography and based on the phytochemical analysis, the fractions enriched with phenolic compounds were isolated and characterized by FT-IR spectroscopy. The IR-spectra have shown the presence of respective functional groups, indicating the isolation of specific phenol enriched compounds from CME which may be further used for drug development. During the evaluation of antidiabetic potential of CME in alloxan induced diabetic rats, the animals showed non-significant ($p>0.05$) increase (2.12%) in observed body weights, however, showed the significant decrease in blood glucose levels (-42.24%, $p<0.001$) compared to the diabetic control rats. Our findings suggest that CME may be used as a potential hypoglycemic herbal medication or might be used in conjunction with the usual synthetic drugs to boost activity and limit the side effects.

Keywords: *Nigella sativa*, *Cicer arietinum*, Anti-hyperglycemic, Antidiabetic, Antioxidant

Citation: Malik Z.; Akhtar MT.; Siddique AB.; Sultana N.; Irfan MI.; Qadir R.; Saleem S.; Shah SNS.; Saadia M.. Hypoglycemic potential of combined methanolic extrac of sativa (black cumin) and Cicer aerietinum (chickpea). *Pakistan Journal of Biochemistry and Biotechnology*, **2021**, 2 (2), 261-. https://doi.org/10.52700/pjbb.v2i2.55

Received: 13-09-2021
Accepted: 28-12-2021
Published: 31-12-2021

1. Introduction

Diabetes is considered a disorder of metabolism, more than a disease [1]. The main cause of diabetes among many is the oxidative stress resulting from various life style alterations

that leads to abnormal metabolism and dysfunctioning of body cells [2]. Oxidative stress accelerates the production of free radicals which are responsible for various types of diseases including diabetic nephropathy, retinopathy and neuropathy with high impact on the quality of life and overall the prospects of life [3]. Many synthetic drugs have been in use to manage the disease including biguanides, alpha glycosidase inhibitors, and sulfonylureas. These drugs have shown the severe adverse effects like hypoglycemia and hepatotoxicity [4]. To overcome these problems, researchers are now using the remedies based on phytochemicals to control side effects. So the antidiabetic potential of several plants and their mechanisms of action have been confirmed in various research investigations [5]. Anti-hyperglycemic effect of plants have been reported mainly due to the presence of different phytochemicals like flavonoids, terpenoids, alkaloids, glycosides or carotenoids [6]. Mostly the plants exert their antidiabetic effect either by decreasing beta cells apoptosis or by repairing insulin secreting pancreatic cells [7]. Due to the reported significant hypoglycemic effects of *Nigella sativa* and *Cicer arietinum* we in present study investigated the antioxidant and hypoglycemic effects of combined methanolic extract of plant seeds, which is not studied so far. Past studies have shown the significant pharmacological importance of seeds of these plants against diverse diseases. *Nigella sativa* is an annual herb belongs to *Ranunculaceae and native to pak-indo region. Phytochemical analysis shows that it contains fixed oil, essential oil, alkaloids, flavonoids, tannins, and saponins* [8]. *Black cumin showed broad spectrum biological activities including hypolipidemic [9], antioxidant, antidiabetic [10], hepatoprotective [11, 12] and nephroprotective [13]. The active ingredient of Nigella sativa thymoquinone showed* anti-diabetic potential in regenerating β-cells of the pancreas and ameliorating pancreatic inflammation and oxidative stress, and highlight its novelty in repressing apoptosis of β-cells and enhancing islet revascularization in STZ-diabetic rats *[14]*. Legumes are a rich source of protein in the human diet and are known best alternative nutritional source for diabetic, cancer, overweight, obesity and cardiovascular diseases patients *[15]*. The phytochemical screening also confirmed the presence of many bioactive components including flavonoids, phenols, tannins, carbohydrates, saponins, sterols, alkaloids, coumarins and lectin. Chickpea showed many biological activities such as antioxidant, antidiabetic [16]. The main objective of the study the preparation of herbal formulation of the above plants, its biochemical characterization of the preparation and evaluate the anti-hyperglycemic activity of herbal formulation to suggest some herbal supplementation to usual medication against diabetes.

2 Materials and Methods

2.1 Plant seeds collection

Seeds of *Nigella sativa* and *Cicer arietinum* were purchased from the local market of Sargodha and authenticated by taxonomist from Department of Botany, University of Sargodha. The combined extract of *Nigella sativa* and *Cicer arietinum* seeds was obtained by maceration process.

2.2 Extraction

All chemicals (reagents and solvents) used were of analytical grade, purchased from Sigma-Aldrich. The combined methanolic extract of seeds of *Nigella sativa* and *Cicer arietinum* was prepared by macerating 1 kg seeds powder of each plant species (1:1) in 20 liter methanol. The maceration process was followed by dividing the mixed seeds powder in eight packets of weighing 250 g each using electric balance. Each packet of mixed seeds powder was dissolved in 834 ml methanol for 24 hours. After through maceration, the filtrate was separated by cheese cloth followed by filtration through Whatmann filter paper No.1. The residues were again macerated for 2nd day by adding 834 ml methanol for next 24 hours. Similarly, the left over residues were macerated 3rd time in 834 ml methanol for next 24 hours. The collected filtrate of each day was combined to one. The same maceration process was repeated for other seven (7) parts of mixed seeds powder, and all the filtrates were then combined to get the maximally enriched methanolic extract [17]. The filtrate was condensed by rotary evaporator (Heidolph Laborota 4000 efficient HB Digital, Germany) to evaporate the solvent at 37°C. Thus a pure mixed methanolic extract of *Nigella sativa* and *Cicer arietinum* seeds was collected named CME. After drying and weighing, the percentage extractability was calculated with formula;

% extractability = wt. of extract obtained/ wt. of plant material (dry wt.) X 100

2.3 Fractionation of Combined Methanolic Extract (CME)

2.3.1 Pre-Thin layer chromatography (TLC)

Table 1: solvent combinations for TLC study of different fractions

Sr. No.	Solvent system	Ratio
1.	n-hexane: chloroform	2:8
2.	n-hexane: chloroform	5:5
3.	n-hexane: chloroform	8:2
4.	diethyl ether: ethyl acetate	2:8
5.	diethyl ether: ethyl acetate	5:5
6.	diethyl ether: ethyl acetate	8:2
7.	ethyl acetate: methanol	2:8
8.	ethyl acetate: methanol	5:5
9.	ethyl acetate: methanol	8:2
10.	chloroform: methanol	2:8
11.	chloroform: methanol	5:5
12.	chloroform: methanol	8:2
13.	n-hexane	1
14.	chloroform	1
15.	diethyl ether	1
16.	ethyl acetate	1
17.	methanol	1

Selected solvent system for fractionation of CME through column chromatography		
1.	n-hexane	1
2.	n-hexane: ethyl acetate	(1:1)
3.	ethyl acetate	1
4.	ethyl acetate: methanol	(1:1)
5.	methanol	1

The Combined methanolic extract was analyzed by pre-TLC (MERCK, Germany; pre-coated silica gel of 0.25 mm layer (70-230 mesh) on aluminum back with fluorescent indicator $60F_{254}$) before column chromatography to select the good solvent combinations for fractionation through column chromatography. The TLC cards (2x4 cm) were cut in pieces. Capillary tube was used to extract spots on TLC card pieces. Based on the polarity ranges different developing solvents and their combinations were used for pre-TLC to achieve a good resolution. The pre-TLC results were observed under the UV Lamp (LAMANG) and marked the spots on TLC plates. Based on the findings of pre-TLC the five solvent systems were selected for fractionation of CME through column chromatography (Table 1).

2.3.2 Column Chromatography:

Fractionation of combined methanolic extract (CME) was done with glass column (720X 42 mm, PYREX) packed with 100 g of silica gel (120-240 mesh, Merck) and 300 ml n-hexane for wet packing of column. A 20 g of CME was loaded on gel and eluted with solvent system (2000mL) in order of increasing the polarities. The fractions were collected at rate of 10 ml/min. Approximately 92 fractions were collected measuring 100 ml each. The each fraction was tested by post-TLC.

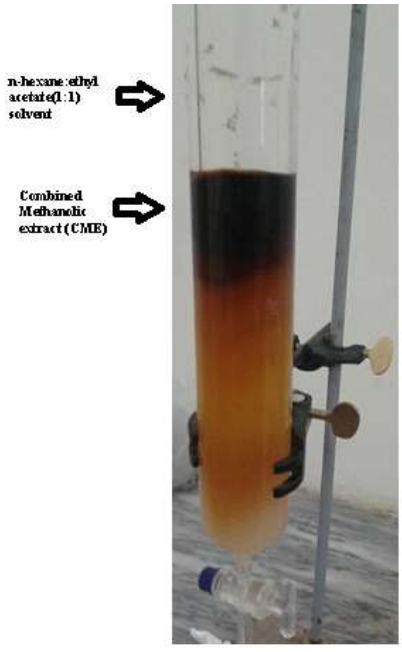


Figure 1. Fractionation of combined methanolic extract by column chromatography by n-hexane:ethyl acetate (1:1) solvent combination

2.3.3 Post Thin Layer Chromatography (TLC):

The eluted fractions were observed for the presence of phytochemicals through post-TLC. Two combination of developing solvents; n-hexane: ethyl acetate (1:1) and ethyl acetate: methanol (1:1) were used for post-TLC. The TLC plates were observed under UV lamp, spots were marked and the solvent front was calculated for R_f values as;

R_f value = Distance moved by the compound(s) (Location by the spot)/ Distance moved by the mobile phase (the solvent front)

2.4 Phytochemical Analysis

The eluted fractions and the pure combined methanolic extract were tested for the presence of phytochemicals using the already determined methods [18].

2.5 Infra-red spectroscopy

The fractions enriched with phenols were selected to confirm their functional groups by IR spectroscopy. Spectra were recorded on IR prestige-21 (200 V) FT-IR spectrometer, SCHIMADZU (Japan), at High-Tech Laboratory, Department of Pharmacy, University of Sargodha. The analysis was done by the KBr discs including analyte.

2.6 Antioxidant Activity of CME

2.6.1 DPPH Assay

Antioxidant activity of CME was analyzed using [19] method of scavenging free radicals by DPPH assay. The DPPH working solution was prepared in methanol (1 mg/mL). Different concentrations (25, 50, 100, 200, 300 and 400 μg/mL) were prepared from stock solutions of CME and the standard (10mg/mL). Then 4 mL of DPPH working solution was added in each test tube and kept in dark for 30 minutes. Absorbance was measured at 517 nm on UV-visible spectrophotometer. Methanol was used as blank and Gallic acid was used as standard antioxidant compound. The radical scavenging activity was measured by calculating IC50 values by using following formula;

$$Percentage\ Inhibition = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ Sample}{Absorbance\ of\ the\ control}$$

2.6.2 Total phenolic contents (TPC)

Total phenolic contents in CME were determined using Folic-Ciocalteu method described by [20]. Stock solution of CME was prepared (2mg/mL). Different dissolutions (10, 20, 30, 40, 50, 100, 150 and 200 ppm) of gallic acid standard were subsequently prepared from stock solution (500 ppm). Distilled water served as blank and gallic acid was used as standard. The reaction mixtures were incubated for 30 min at room temperature. The absorbance was noted at 700 nm using spectrophotometer. The results were expressed in terms of gallic acid equivalent (mg gallic acid/g of dry mass), a common reference compound.

2.7 Hypoglycemic potential of CME

Albino rats (260-340 g body weight) were taken from the animal house of veterinary institute of research Lahore (VIR), Punjab, Pakistan. The animals were given free access to food and water and acclimatized at 24 ± 1°C temperature for familiarization before experimental trial. Animals were used for the antidiabetic potential of CME. The animals were divided into three groups as follows;

Group A: served as control group received normal saline solution

Group B: served as diabetic control group received alloxan in normal saline solution

Group C: served as treatment group received alloxan + CME

Each group contains three albino rats. Diabetes was induced in animals of group B and C by a single intra-peritoneal injection of 10% alloxan (150 mg/kg, Applichem-Germany) freshly dissolved in 0.9% of normal saline solution to induce diabetes at the start of the experiment [21]. Animals were fasted for 14 hours earlier to injection of alloxan. The control group was injected with the same volume of normal saline solution. Three days after alloxan induction diabetes was confirmed by the hyperglycemia through glucometer (On Call EZ II, ACON® Laboratories, Inc., USA). The treatment group received the oral dose of CME (200 mg/kg b.w) [22] daily for 14 days. Body weight of animals was measured at the start and end of the study trial. At the end of the experiment, rats in all groups were fasted for 12 hours and blood glucose level was checked from the blood samples obtained from tail by puncturing through needle.

2.8 Statistical analysis

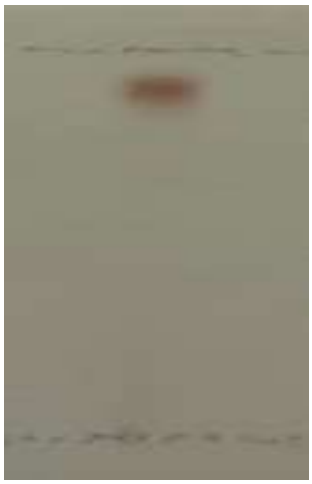
Results are presented as mean ± standard deviation. Statistical analysis was carried out with one-way ANOVA analysis and Tukey's post hoc test for multiple comparisons (differences among means), using Statistical Package for Social Sciences (SPSS) software, version 21.0. Differences were considered significant at $p < 0.05$.

Table 2. Concentration of Plant Contents in Different Extracts/fractions.

Combined/Plant Extract	Quantity
Seed powder maceration (2 kg/ 20L methanol)	100g/L (0.1 g/mL)
Extractability (after rotary)	48 g extract/100 g crude
CME loaded on silica gel	20 g
100 mL of each fraction (contains plant contents (total 92 fractions)	0.217 g (217 mg)

For fractionation, the CME was tested through Pre-TLC for approximate solvent choices. Based on the Pre-TLC tests, The CME mixture (20 g) was fractionated through five solvents/combinations. Total of 92 fractions (100mL each) were collected at rate of 10 mL/min containing 217 mg of phytocontents in each fraction (Table 1). The fractions were observed by using two developing solvents for post-TLC hexane: ethyl acetate (1:1) and methanol: ethyl acetate (1:1). The appearance of spots with different colors indicated the presence of different types of phytochemicals. Representatives are shown in figure 2. Different solvents/combinations with the respective R_f values are presented in table 3.

Fraction 7 of methanol (100%) in developing solvent methanol: ethyl acetate (5:5). The presence of a pink colored spot indicates the existence of anthroquinone



Fraction 2 of methanol (100%) in developing solvent system methanol: ethyl acetate (5:5). The presence of a brown colored spot indicates the existence of tannins

Fraction 3 of methanol (100%) in developing solvent system methanol: ethyl acetate (5:5). The presence of a purple colored spot indicates the existence of saponins

Fraction 2 of ethyl acetate (100%) in developing solvent system methanol: ethyl acetate (5:5) The presence of a yellow colored spot indicates the existence of alkaloid

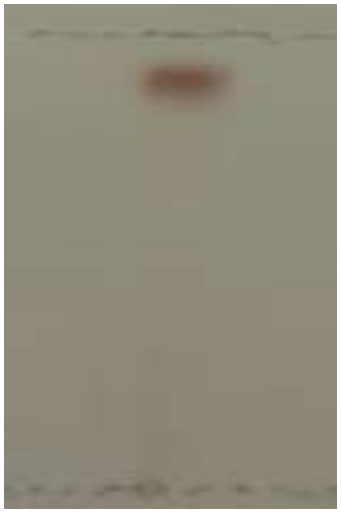
Fraction 8 of ethyl acetate (100%) in developing solvent system methanol: ethyl acetate (5:5). The presence of a blue colored spot along with the yellow one indicates the existence of phenolics along with alkaloids.

Fraction 1 of ethyl acetate (100%) in developing solvent system methanol: ethyl acetate (5:5). The presence of a brown colored spot indicates the existence of tannins

Fraction 2 of ethyl acetate: methanol (50:50) in developing solvent system hexane: ethyl acetate (5:5). The presence of a yellow colored spot indicates the existence of alkaloid

Fraction 16 of ethyl acetate: methanol (50:50) in developing solvent system hexane: ethyl acetate (5:5). The presence of a yellow colored spot indicates the existence of alkaloid

Fraction 3 of methanol (100%) in developing solvent system hexane: ethyl acetate (5:5). The presence of a yellow colored spot indicates the existence of alkaloid



Fraction 8 of methanol (100%) in developing solvent system hexane: ethyl acetate (5:5). The presence of a brown colored spot indicates the existence tannins

Fraction 4 of methanol (100%) in methanol: ethyl acetate (5:5). The presence of a yellow colored spot along the blue colored indicates the existence Phenols & alkaloids

Fraction 14 of methanol (100%) in methanol: ethyl acetate (5:5). The presence of a yellow colored spot along the blue colored indicates the existence Phenols & alkaloids

Figure 2. The post TLC results of some representative fractions.

Table 3. Determination of Rf Values of Different Fractions from CME

Sr. No.	Solvent systems	Fractions	Developing solvent	Rf values
1	n-hexane (1)	F7	Methanol: ethyl acetate (1:1)	0.863
2	n-hexane: ethyl acetate (1:1)	F1	n-hexane: ethyl acetate (1:1)	0.827
		F6	n-hexane: ethyl acetate (1:1)	0.944
3	Ethyl acetate (1)	F2	n-hexane: ethyl acetate (1:1)	0.829
		F8	Methanol: ethyl acetate (1:1)	0.878
4	Ethyl acetate: methanol (1:1)	F1	n-hexane: ethyl acetate (1:1)	0.636
		F2	n-hexane: ethyl acetate (1:1)	0.727
		F16	Methanol: ethyl acetate (1:1)	0.861
5	Methanol (1)	F3	n-hexane: ethyl acetate (1:1)	0.906
		F8	n-hexane: ethyl acetate (1:1)	0.871
		F4	Methanol: ethyl acetate (1:1)	0.891
		F14	Methanol: ethyl acetate (5:5)	0.902

Solvent	**Tests**										
Methanol (100 %)	**Alkaloids**	**Flavonoids**	**Phenols**	**Saponins**	**Tannins**	**Terpenoids**	**Quinones**	**Anthocyanin**	**Proteins**	**Carbohydrates**	**Anthraquinones**
Fraction 1	+++	-	++	-	++	-	++	-	+	+++	-
Fraction 2	+++	-	++	-	++	-	+	-	-	++	-
Fraction 3	+++	-	+++	-	+++	-	-	-	-	+++	-
Fraction 4	+++	-	+++	-	+++	-	-	-	+++	+++	-
Fraction 5	+++	-	++	-	++	-	-	-	++	++	-
Fraction 6	+++	-	++	-	++	-	+	-	+++	+++	-
Fraction 7	+++	-	+++	-	+++	-	-	-	+++	+++	-
Fraction 8	+++	-	++	-	++	-	-	-	-	+++	-
Fraction 9	+++	-	+++	-	+++	-	-	-	-	+++	-
Fraction 10	+++	-	+++	-	+++	-	-	-	-	+++	-
Fraction 11	+++	-	+++	-	+++	-	-	-	++	+++	-
Fraction 12	+++	-	+++	-	+++	-	-	-	++	+++	-

Fraction 13	+++	-	++	-	++	-	-	-	+	++	-
Fraction 14	+++	-	+++	-	+++	-	-	-	++	+++	-
Fraction 15	+++	-	++	-	++	+	-	-	+	++	-
Fraction 16	+++	-	+++	-	+++	+	-	-	+	+++	-
Fraction 17	+++	-	+++	-	+++	+	-	-	-	+++	-
Fraction 18	+++	+	++	-	++	+	-	-	++	+++	-
Fraction 19	+++	-	+++	-	+++	-	-	-	++	+++	-
Fraction 20	+++	-	+++	-	+++	-	-	-	+++	+++	-

3.2

Qualitative Phytochemical Analysis

The CME mixture and the column eluted fractions were tested for the presence of phytochemicals including; alkaloids, flavonoids, phenols, saponins, tannins, terpenoids, quinones, anthocyanin, anthraquinones, carbohydrates and protein using standard methods (table 4-5).

Table 4. **Qualitative Phytochemical Analysis of a Representative Fraction (methanol 100%):** '+++' shows strong presence, '++' shows moderately presence, '+'shows normal presence and "-" shows the absence of respective phytochemicals.

Table 5. Qualitative Phytochemical Analysis of CME

Solvent	Tests										
Combined methanolic extract (*Nigella sativa* + *Cicer arietinum*)	**Alkaloids**	**Flavonoids**	**Phenols**	**Saponins**	**Tannins**	**Terpenoids**	**Quinones**	**Anthocyanin**	**Proteins**	**Carbohydrates**	**Anthraquinones**
	+++	++	+++	+++	+++	++	++	-	+++	+	-

3.3 FT-IR spectroscopy:

Most of the column isolated fractions of CME have confirmed the presence of phenolic compounds. The characteristic peaks of aromatic ring (1420-1500 cm-1), carbonyl group of carboxylic acid (1720-1780 cm-1), broad peak of hydrogen bonded hydroxyl groups of phenols (3200-3400 cm-1), free hydroxyl group of phenolic compound (3600-3750 cm-1) and alkyl groups (2900 cm-1) confirmed the presence of phenolic content in different fractions. Moreover, spectra of some fractions [(F3 and F18 from Ethyl acetate: Methanol (1:1), F1 and F3 from Ethyl acetate (1) and F15 from (Hexane (1)] also showed the peaks of nitrile (2333-2345 cm-1) and/or of primary and secondary amine groups (3200-3450 cm-1) indicating the presence of alkaloids contained within (Table 6).

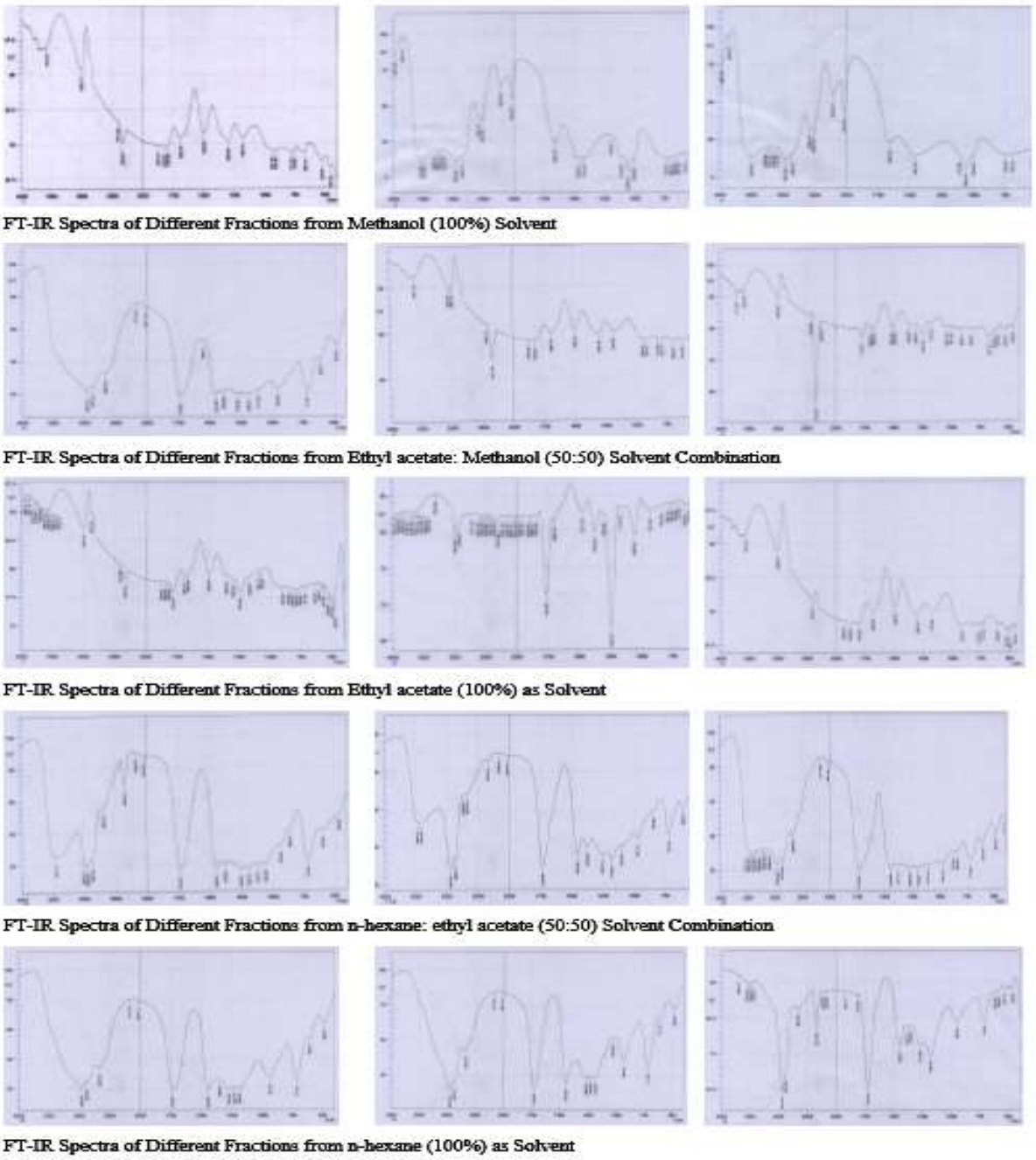


Figure 3: FT-IR spectra of different fractions developing in different solvent systems

Table 6. FT-IR Identification of Functional Groups for Presence of Phytochemicals in Selected Fractions

Sr. No.	Solvents/Combinations	Fractions	Peak values (cm^{-1})	Functional group

1.	**Methanol (1)**	F1	2999	C-H stretching of alkyl group
			1494	C-C stretching of aromatic ring
			3570-3700	O-H stretching of free hydroxyl group of phenolics
			1665	C=O stretching of carbonyl group
		F6	2968	C-H stretch of alkyl group
			2592	C-H stretch of aldehyde group
			1463	C-C stretching of aromatic ring
			1636	C=O stretch of aldehyde group
			2800-3600	O-H stretching of hydrogen bonded hydroxyl group of phenolics
		F18	2964	C-H stretch of alkyl group
			2852	C-H stretch of aldehyde group
			1458	C-C stretching of aromatic ring
			2800-3600	O-H stretching of hydrogen bonded hydroxyl group of phenolics
			1636	C=O stretch of aldehyde group
2.	**Ethyl acetate: Methanol (1:1)**	F2	2954	C-H stretch of alkyl group
			1444	C-C stretching of aromatic ring
			1734	C=O stretching of carboxylic acid
			2600-3500	O-H stretching of hydrogen bonded hydroxyl group of phenolics
		*F3	1492	C-C stretching of aromatic ring
			1685	C=O stretching of carbonyl group of ketone
			2341	Nitrile group of alkaloids
			3005	C-H stretching of alkyl group
		*F18	1500	C-C stretching of aromatic ring
			1774	C=O stretching of carbonyl group of ester
			2345	Nitrile group of alkaloids
			2989	C-H stretching of alkyl group
			3576, 3714	N-H stretching of primary amine
3.	**Ethyl acetate (1)**	*F1	1494	C-C stretching of aromatic ring
			1776	C=O stretching of carbonyl group of ketone
			2333	Nitrile group of alkaloids
			2995	C-H stretching of alkyl group

			3572, 3776	N-H stretching of primary amine
		*F3	1471	C-C stretching of aromatic ring
			1762	C=O stretching of carbonyl group of ketone
			2335	Nitrile group of alkaloids
			2983	C-H stretching of alkyl group
			3670, 3790	N-H stretching of primary amine
		F18	1490	C-C stretching of aromatic ring
			1683	C=O stretching of carboxylic acid
			3008	C-H stretching of alkyl group
			3564-3700	O-H stretching of free hydroxyl group of phenolics
4.	**Hexane: Ethyl acetate (1:1)**	F1	1446	C-C stretching of aromatic ring
			1734	C=O stretching of carboxylic acid
			2985	C-H stretching of alkyl group
			3250-3441	O-H stretching of free hydroxyl group of phenolics
		F13	1452	C-C stretching of aromatic ring
			1732	C=O stretching of carboxylic acid
			2939	C-H stretching of alkyl group
			3200-3446	O-H stretching of free hydroxyl group of phenolics
		F17	1444	C-C stretching of aromatic ring
			1735	C=O stretching of carboxylic acid
			2956	C-H stretching of alkyl group
			2850-3450	O-H stretching of free hydroxyl group of phenolics
5.	**Hexane (1)**	F1	1444	C-C stretching of aromatic ring
			1735	C=O stretching of carboxylic acid
			2956	C-H stretching of alkyl group
			2600-3400	O-H stretching of free hydroxyl group of phenolics
		F2	1444	C-C stretching of aromatic ring
			1728	C=O stretching of carboxylic acid
			2951	C-H stretching of alkyl group
			2700-3420	O-H stretching of free hydroxyl group of phenolics
		*F15	1446	C-C stretching of aromatic ring
			1722	C=O stretching of carbonyl group of ketone

			2341	Nitrile group of alkaloids
			2931	C-H stretching of alkyl group
			3458	N-H stretching of secondary amine

*Fractions indicating the presence of alkaloids.

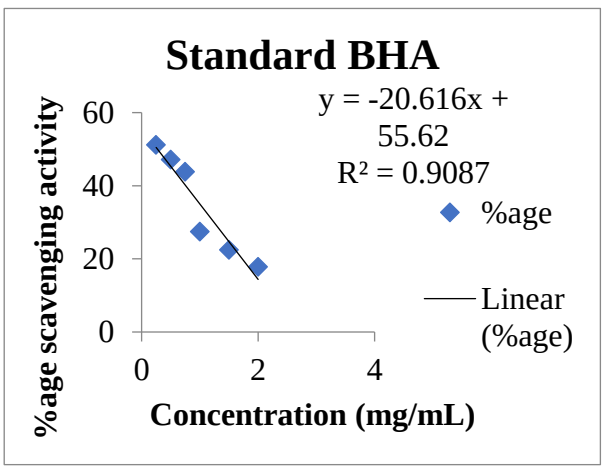
3.4 Antioxidant Potential:

Antioxidant potential of CME was determined through DPPH assay and determining the total phenolic contents (TPC). Significant ($p<0.001$) antioxidant activity was exhibited by all concentrations (0.25-2 mg/ mL) of sample compared to that of butylated hydroxyanisole (BHA). The values of absorbance gradually increased as dissolution of standard and sample extract increases. Linear calibration curve of standard (BHA) and sample extract was plotted (Figure 3). The maximum scavenging activity of standard and sample extract was "51.18%" and "49.51%" respectively.

Table 7. DPPH Scavenging Activity and Percentage Scavenging Activity of Standard and CME

Sr. No.	Concentration (mg/mL)	Absorbance of standard (517 nm) (Mean ± SD)	Absorbance of sample extract (517 nm) (Mean ± SD)	Scavenging activity of standard (%)	Scavenging activity of sample extract (%)
1	0.25	0.1694±2.27	0.1752±2. 21[a]	51.18	49.51
2	0.5	0.1834±1.13	0.2002±3.18[a]	47.13	42.30
3	0.75	0.1948±3.22	0.2116±1.33[a]	43.86	39.02
4	1	0.2515±2.42	0.2602±2.19[a]	27.50	25.01
5	1.5	0.2689±1.28	0.2890±2.44[a]	22.49	16.71
6	2	0.2850±3.31	0.3160±2.41[a]	17.86	8.93

Table 7: Values are means± SD (n3). [a] $p<0.001$ comparison to standard butylated hydroxyanisole (BHA). The data were analyzed using One-way-ANOVA analysis and Tukey's post hoc test.



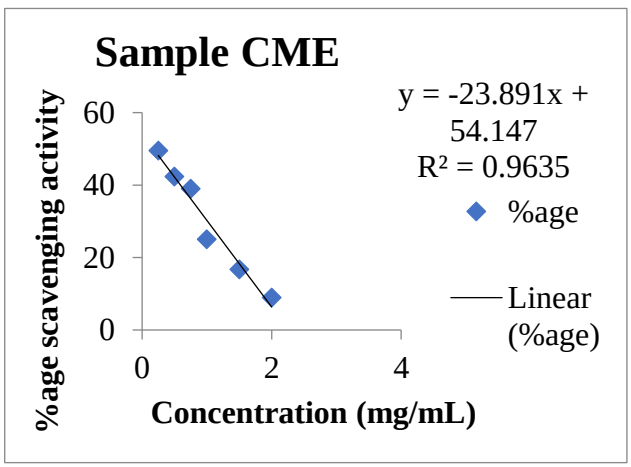
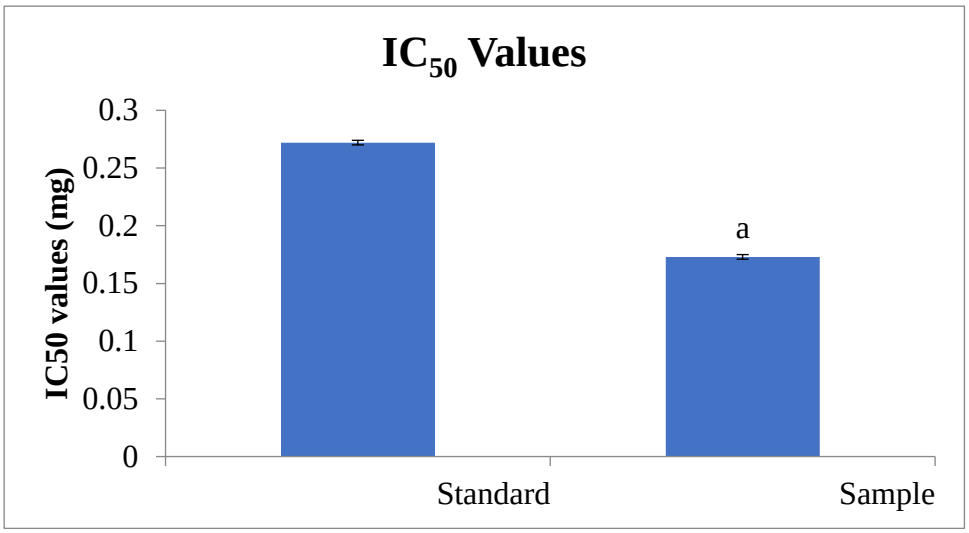


Figure 4: Linear Calibration Curve of Butylated Hydroxyanisole (BHA) and CME

The IC_{50} values showed that the CME possessed significant ($p<0.001$) DPPH free radical scavenging activity which was (36.39%) than standard BHA [figure 5].

Figure 5. IC_{50} Values of Standard and CME in DPPH Assay: The IC 50 values for



standard and CME were 0.272 mg and 0.173 mg respectively ([a] $p<0.001$). The data were analyzed using one way-ANOVA analysis and Tukey's post hoc test.

3.4.1 Total phenolic contents (TPC)

Phenols are important compounds that are mostly present in all species of plants and have medicinal value as they increase the rate of scavenging of oxidants axting as antioxidants. Total phenolic content has been determined by Folic-Ciocalteu method and the gallic acid was used as standard. The results have shown the significant ($p<0.001$) contents of total phenolics in CME compared to the standard. From the caliberation curve of gallic acid the TPC value in sample was noted 254.2 mg GAE/ g dry wt. of sample [figure 6].

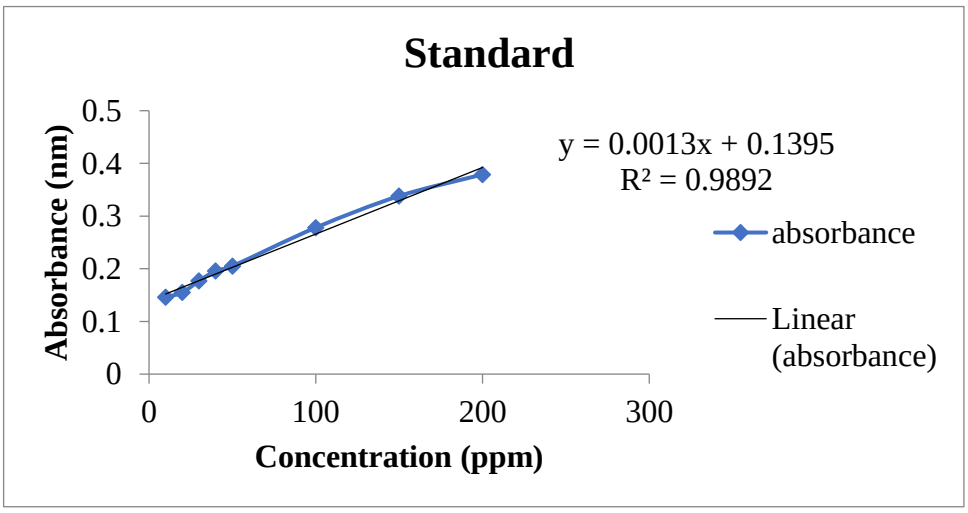


Figure 6. Linear Calibration Curve of Gallic Acid

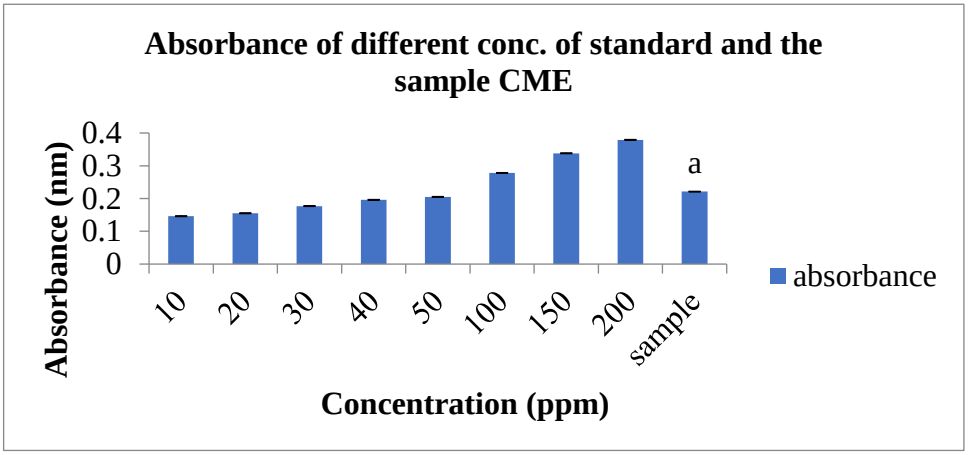


Figure 7. Total Phenolic Contents (TPC) of Standard and CME: comparison of CME to gallic acid. The data were analyzed using one way-ANOVA analysis and Tukey's post hoc test ([a] $p<0.001$).

3.5 Effect of CME on Body Weight

Non-significant ($P< 0.001$) effect of CME administration on mean body weight diabetic rats was noted as compared to the animals of diabetic control group. The animals of diabetic control group also showed the non-significant reduction in body weight as compare to control group (normal) animals. When these diabetic rats were treated with CME, the mild increase in body weight was observed as compared to the diabetic control group but was not significant at the 14th day of experiment (Table 8).

Table 8: Effect of CME on Body Weight

Parameters	**Values (Mean±S.D.)**		
	Normal control group	Diabetic control group	CME treated group
Body weight (g)	334±3.52	330±3.21^{ns}	337±3.4^{ns}

Effect of CME Administration on Mean Body Weight: Values are expressed as mean ± SD ($n = 3$). [ns] non-significant comparison of CME to diabetic control group and with normal control group. The data were analyzed using one way-ANOVA analysis and Tukey's post hoc test.

3.6 Effect of CME Administration on Mean Blood Glucose Level (BGL)

Significant ($P< 0.001$) increase in mean blood glucose levels was noted in animals of diabetic control group after the induction of alloxan injection. However, in alloxan + CME treated group gradual and significant ($P< 0.001$) reduction in BGL was noted on 14th day as compared to the diabetic control group [table 9].

Table 9: effect of CME on blood glucose levels

Parameters	**Values (Mean±S.D.)**		
	Control group	Diabetic control group	CME treated group
	95±2.08	300±3.1[a]	160.2±3.02[b]

Blood glucose level (mg/dL)			

Effect of CME on Blood Glucose Level: Values are expressed as mean ± SD (n = 3) [a] Significant comparison of diabetic control group to control group, and [b] significant comparison of CME treated group to the diabetic group. The data were analyzed using one way-ANOVA analysis and Tukey's post hoc test.

4. Discussion

By using the TLC technique to detect presence of specific compound(s) at very initial steps has various advantages like lower cost, short-time analysis, the possibility of multiple detections, and specific derivatization on the same plate [27]. Thus, TLC profiling showing different Rf values in different solvents describes the polarity of the isolated compounds and also directs towards the selection of the appropriate solvent system to isolate the pure compound(s) through column chromatography. Giri et al., (2020) demonstrated the presence of bioactive compounds including flavonoids, saccharides, phenols and tannins in plant samples screened through TLC profiling [28]. The [29] also related the color of an isolated compound on TLC plate with the class of the phytochemicals. They used three (3) solvent system [petroleum ether: Ethyl acetate: Methanol (6:2:2)] for phytochemicals isolation and found that the fractions of methanol extract (F16 and F17) produced deep blue black color indicating the presence of phenols. The fractions F24 and F25 showed orange precipitate which indicated the presence of alkaloids, fractions F19 and F20 gave intense green color showing the presence of flavonoids, and reddish brown color was noted for fractions F4 and F5 indicating the presence of steroids.

Phytochemical screeninig showed that Anthocyanin and anthraquinones were not observed in pure combined methanolic extract (CME) and from all the other eluted fractions as well. However, the other phyto-constituents were present according to the nature (polarity) of the solvent systems used as also described in earlier study [30]. We found flavonoids as moderately present in CME. The researcher [31] also reported the moderate existence of flavonoids present in methanolic and ethanolic extract of *Nigella sativa* seeds while performing the in-vitro antibacterial activity of these extracts. However, we noted the strongly presence of phenols in CME , while in an another study [32] reported the low amount of phenolics and flavonoids in aqueous extract of *Nigella sativa*. Similarly, the presence of flavonoids and phenols in different solvents (ethanol, chloroform, hexane, ethyl acetate and aqueous) was also reported in seed extracts of *Cicer arietinum* [33, 34].

From the findings of FT-IR spectra, it is inferred that methanol has been the good choice for separation of most of the phenolic compounds, while ethyl acetate may be preferred to isolate alkaloids form the plant tissues. The [35] has identified the major bioactive components by FT-IR of aqueous extract of *Cicer arietinum* seeds. The FT-IR analysis showed that the presence of C-H (2960 cm-1), O-H (3443-3414 cm-1), C=O (1672-1638 cm-

1) and C-O (1027-1024 cm-1) stretching vibration bands. Similarly, [36] have identified the bioactive constituents in ethanolic extract of *Nigella sativa* through phytochemical analysis, Gas Chromatography-Mass Spectroscopic (GC-MS) and Fourier Transform Infrared Spectroscopy (FT-IR). The FT-IR analysis showed the presence of functional groups; alkanes (2927.80, 2854.20 and 1464.50 cm-1), acids (1713.00, 1244.00 cm-1), ester (1185.30 cm-1), alky1 (1046.52 cm-1) and alkenes (720.75-918.43 cm-1). We thus isolated the phenolic rich fractions through column chromatography and the presence of specific phenolic groups was characterized by FT-IR spectroscopy. Further studies on isolations of some specific phenolic groups as 'bioactives' from such fractions would be of much medicinal value.

Earlier, [23] has also reported the significant ($p<0.05$) DPPH radical scavenging activity (51.18%) of *Cicer arietinum* aqueous ethanolic seed extract. Past studies also revealed that the administration of plant extracts as antioxidants ameliorates the severity of the disease [23]. The [37] described that the administration of ethanolic extract of *Nigella sativa* showed significant showed antioxidant activity in indomethacin treated rats. In another [38] reported the significant antioxidant effect of hydroethanolic extract of *Nigella sativa* on lipoplysaccarides induced inflammation and oxidative stress in rats. Earlier researcher also determined the TP contents in plant extracts to evaluate their antioxidant potential. The study conducted by [39] reported total phenolic contents (8.45 ± 1.81) in methanolic extract of *Nigella sativa*. Also [40] has reported the TPC of desi and kabuli chickpea aqueous methanolic extract of raw seeds of *Cicer arietinum* as 203-255 and 101-178 mg/100 g of the dry weight.

Injection of alloxan to rats induce sudden loss of weight, and increase a blood glucose level by the destruction of beta pancreatic cells [41]. In present study the administration of CME has significantly decreased the blood glucose level of diabetic rats. Our result was consistent with the study by [10] who described the significant reduction in BGL in alloxan induced diabetic rats after administration of *Nigella sativa* oil to the animals. Also effective results were reported by [42] who showed that the aqueous extract of *Nigella sativa* induced hypoglycemic and antioxidant effects in STZ induced diabetic rats by increasing the expression of Bcl2, CAT, SOD, GPX, GST and IGF-1 genes. [25] reported the effectiveness of ethanolic extract of *Cicer arietinum* in inducing gradual increase in body weight and lowering the blood glucose levels (BGL) in STZ induced diabetic rats [25]. In another study [43] have also reported the beneficial effect of *Cicer arietinum* on body weight and BGL of the alloxanized diabetic rats.

5. Conclusions

To limit the severity of synthetic drugs to manage diabetes, the ethno-botanical and traditional uses of natural compounds specially of plant origins has received much attention and studies are progressing in increasing the effectiveness and safety of herbal remedies using different strategies including choice of the good plant material and the solvents system for better bioactives isolation. In this study the combined methanolic extract (CME) of seed powder of *Nigella sativa* and *Cicer arietinum* was selected to analyze the combined antioxidant and hypoglycemic effect of these two, which was not studied so far. Different solvents and their combinations (solvent system) were

used to isolate the maximum of phytochemicals from the CME mixture. The qualitative phytochemical analysis revealed the presence of nearly all t medicinally important phytochemical constituents in CME and in all the column eluted fractions. From the findings of FT-IR spectra, it was inferred that phenolics were present in all the solvent systems, so methanol was a better choice to separate most of the phenolic compounds, while ethyl acetate may be preferred to isolate alkaloids form the plant seeds. TLC profiling directed the isolation of specific bioactives and also provided valuable clue regarding the selection of the solvent system according to the nature of the phytochemicals. Finally, the formulated herbal remedy CME showed significant antioxidant activities as determined by DPPH assay and the presence of total phenolic contents. The significant hypoglycemic activity determined its potential antidiabetic effects in rats. For future studies it is suggested that the isolation and identifications of fractions enriched with phenolics may be helpful in developing new herbal drugs to cure diabetes.

Author Contributions: For research articles, all the authors showed different services for the completion of research.

Funding: "This research received no external funding"

Acknowledgments: The special thanks to biochemistry and analytical chemistry labs and assistant of lab who provide all equipment's and their services.

Conflicts of Interest: "The authors declare no conflict of interest

References

1. IDF, International Diabetes Federation Atlas, IDF, Aarhus, Denmark, 2019.
2. Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K. B., & Martín, C. 2020. Pathophysiology of type 2 diabetes mellitus. *International journal of molecular sciences*, *21*(17), 6275
3. Faselis, C., Katsimardou, A., Imprialos, K., Deligkaris, P., Kallistratos, M. and Dimitriadis, K., 2020. Microvascular complications of type 2 diabetes mellitus. *Current Vascular Pharmacology. 18*(2): 117-124.
4. Babu, N.R. 2016. Studies on the evaluation of antidiabetic and antioxidant activities using some selected medicinal plants. Int. J. Herb. Med., 4: 21-24.
5. Salehi, B., Ata, A., V Anil Kumar, N., Sharopov, F., Ramírez-Alarcón, K., Ruiz-Ortega, A., & Sharifi-Rad, J. 2019. Antidiabetic potential of medicinal plants and their active components. *Biomolecules. 9*(10): 551.
6. Bouyahya, A., El Omari, N., Elmenyiy, N., Guaouguaou, F. E., Balahbib, A., Belmehdi, O., & Bakri, Y. (2021). Moroccan antidiabetic medicinal plants: Ethnobotanical studies, phytochemical bioactive compounds, preclinical investigations, toxicological validations and clinical evidences; challenges, guidance and perspectives for future management of diabetes worldwide. *Trends in Food Science & Technology*.
7. Malviya, N., Jain, S. and Malviya, S. 2010**.** Antidiabetic potential of medicinal plants. *Acta Pol Pharm.*, 67(2), 113–118.
8. Ali, B. H., & Blunden, G. 2003. Pharmacological and toxicological properties of Nigella sativa. *Phytotherapy Research: An international journal devoted to pharmacological and toxicological evaluation of natural product derivatives*, *17*(4): 299-305.

9. Yahia, S., Benomar, S., Dehiba, F., Allaoui, A., Guillen, N., Rodriguez-Yoldi, M.J., Osada, J. and Boualga, A. 2017. Hypocholesterolaemic and antioxidant efficiency of chickpea (Cicer arietinum) protein hydrolysates depend on its degree of hydrolysis in cholesterol-fed rat. *Nutrition & Food Science*.

10. Akhtar, M.T., Qadir, R., Bukhari, I., Ashraf, R.A., Malik, Z., Zahoor, S., Murtaza, M.A., Siddique, F., Shah, S.N.H. and Saadia, M. 2020. Antidiabetic potential of Nigella sativa L seed oil in alloxaninduced diabetic rabbits. *Tropical Journal of Pharmaceutical Research*, *19*(2): 283-289.

11. Saadia, M., Sher, M., Bashir, S., Murtaza, M.A., Shah, A. and Khan, M.A. 2019. Comparative hepatoprotective effect of Nigella sativa pre-and post-treatment to rabbits. *Pakistan journal of pharmaceutical sciences*, *32*(1).

12. Majeed, M., Hussain, A.I., Anwar, H., Irfan, S., Chatha, S.A.S., Ali, Q., Mukhtar, I. and Hafeez, Z. 2020. Hepatoprotective effect of desi and kabuli cultivars of Cicer arietinum L (chick peas) against carbon tetrachloride-induced toxicity in rats. *Tropical Journal of Pharmaceutical Research*, *19*(3): 609-615.

13. Ashraf, A., Hassan, F., Batool, S., Nadeem, M., Irshad, M., Siddique, A., Anwar, F., Rubab, S.L., Khaliq, K., Akhtar, M.T. and Akram, N.A. 2020. Protective Effect of Silybum marianum and Nigella sativa Oil Extracts against Cisplatin Induced Nephrotoxicity in Mice. *Current Topics in Nutraceutical Research*, *18*(2).

14. Almatroodi, S. A., Alnuqaydan, A. M., Alsahli, M. A., Khan, A. A., & Rahmani, A. H. 2021. Thymoquinone, the Most Prominent Constituent of Nigella Sativa, Attenuates Liver Damage in Streptozotocin-Induced Diabetic Rats via Regulation of Oxidative Stress, Inflammation and Cyclooxygenase-2 Protein Expression. *Applied Sciences*, *11*(7): 3223.

15. López-Barrios, L., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. 2014. Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. *Journal of food science*, *79*(3): 273-283.

16. Bhagyawant, S.S., Narvekar, D.T., Gupta, N., Bhadkaria, A., Gautam, A.K. and Srivastava, N. 2019. Chickpea (Cicer arietinum L.) Lectin Exhibit Inhibition of ACE-I, α-amylase and α-glucosidase Activity. *Protein and peptide letters*, *26*(7): 494-501.

17. Abdurohaman, Y.A. **2015.** Phytochemical extraction and screening of bioactive compounds from black cumin (*Nigella sativa*) seeds extract. *Am J Life Sci***,** 3(5): 358-364.

18. Harborne JB 1973. Phytochemical methods, London. Chapman and Hall, Ltd. 49-188.

19. Brand-Williams, W., Cuvelier, M.-E., and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food science and Technology *28*: 25-30

20. McDonald, S., Prenzler, P. D., Antolovich, M., & Robards, K. 2001. Phenolic content and antioxidant activity of olive extracts. *Food chemistry*, *73*(1): 73-84.

21. Chaturvedi, S., Sharma, P. K., Garg, V. K., & Bansal, M. 2011. Role of nutraceuticals in health promotion. *Int J PharmTech Res*, *3*(1): 442À8.

22. Malviya, N., Jain, S., & Malviya, S. A. P. N. A. (2010). Antidiabetic potential of medicinal plants. *Acta pol pharm*, *67*(2): 113-118.

23. Narayanan, B. S., & Smrithi, R. K. 2015. DPPH scavenging activity of *Cicer arietinum* seed extract. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *6*(2): 1080-1083.

24. Asaduzzaman, M., Nahar, L., Rahman, S., Hasan, M., Khatun, A., Tamanna, Z., & Alam, M. 2015. Hypoglycemic and hypolipidemic potential of *Nigella sativa* L. seed extract in streptozotocin (STZ)-induced diabetic rats. *Journal of Plant Biochemistry & Physiology*.

25. Bhowmik, A., Mossihuzzaman, M., Kabir, Y. and Rokeya, B. 2016. Glycemic, Insulinemic, Lipidemic and Antioxidant Status of STZ Rats after Chronic Administration of *Cicer arietinum* extract. *Metabolomics: Open Access*, *6*(3): 1-7.

26. Quintero-Soto, M. F., Chávez-Ontiveros, J., Garzón-Tiznado, J. A., Salazar-Salas, N. Y., Pineda-Hidalgo, K. V., Delgado-Vargas, F., & López-Valenzuela, J. A. 2021. Characterization of peptides with antioxidant activity and antidiabetic potential obtained from chickpea (*Cicer arietinum* L.) protein hydrolyzates. *Journal of Food Science*.

27. Senguttuvan, J., & Paulsamy, S. 2014. Thin layer chromatographic analysis for various secondary metabolites in the methanolic root and leaf extracts of *Hypochaeris radicata* L. *American journal of pharmtech research*, *4*(2): 145-156.

28. Giri, S., Giri, U., Subedi, K., Magar, K. T., Pant, S., & Joshi, K. R. 2020. Thin Layer Chromatography (TLC) Based Chemical Profiling and Antioxidant Activity of Selected Nepalese Medicinal Plants. *Journal of Health and Allied Sciences*, *10*(2): 15-22.

29. Yessuf, A. M. (2015). Phytochemical extraction and screening of bio active compounds from black cumin (Nigella sativa) seeds extract. *American Journal of Life Sciences*, *3*(5): 358-364.

30. Tafesse, T.B., Hymete, A., Mekonnen, Y. and Tadesse, M., 2017. Antidiabetic activity and phytochemical screening of extracts of the leaves of *Ajuga remota Benth* on alloxan-induced diabetic mice. *BMC complementary and alternative medicine*, *17*(1): 243.

31. Ishtiaq, S., Ashraf, M., Hayat, M. Q., & Asrar, M. 2013. Phytochemical analysis of *Nigella sativa* and its antibacterial activity against clinical isolates identified by ribotyping. *International Journal of Agriculture and Biology*, *15*(6).

32. Sheriff, M. A., Azmathullah, N. M., & Mohideen, A. S. 2015. Evaluation of Phytochemical and Anti-Bacterial Activity of *Nigella Sativa* L. *The Scitech Journal*, *2*: 23-26.

33. Kamal, A. I. S. H. A., & Ahmad, I. Z. (2014). Phytochemical studies of different phases of germination of *Nigella sativa* Linn—A medicinally import-ant plant. *methods*, *9*(10): 11-12.

34. Alamgir, A. N. M., & Fatema, K. 2013. Phytochemical screening of some antidysenteric medicinal plants of Bangladesh. *Journal of the Asiatic Society of Bangladesh, Science*, *39*(2): 139-146.

35. Narain, K., & Yunus, M. 2015. Quantitative and Qualitative Changes in Seeds of Cicer arietinum L. Applied with Distillery Effluent. *World Applied Sciences Journal*, *33*(8): 1258-1266.

36. Nivetha, K., & Prasanna, G. 2016. GC-MS and FT-IR analysis of *Nigella sativa* L. seeds. *Int J Adv Res Biol Sci*, *3*: 45-54.

37. Paseban, M., Niazmand, S., Soukhtanloo, M., & Meybodi, N. T. 2019. The preventive effect of *Nigella sativa* seed on gastric ulcer induced by indomethacin in rat. *Journal of Herbmed Pharmacology*, *9*(1), 12-19.

38. Mokhtari-Zaer, A., Norouzi, F., Askari, V.R., Khazdair, M.R., Roshan, N.M., Boskabady, M., Hosseini, M. and Boskabady, M.H., 2020. The protective effect of *Nigella sativa* extract on lung inflammation and oxidative stress induced by lipopolysaccharide in rats. *Journal of Ethnopharmacology*, *253*: 112653.

39. Wijewardhana, U., Gunathilaka, U. G. S. A., & Navaratne, S. 2019. Determination of total phenolic content, radical scavenging activity and total antioxidant capacity of cinnamon bark, black cumin seeds and garlic. *International Research Journal of Advanced Engineering and Science*, *4*(2): 55-57.

40. Shruti, S., Neelam, Y., Alka, S., & Rajendra, K. 2013. Antioxidant activity, nuetraceutical profile and health relevant functionality of nine newly developed chickpea cultivars (Cicer arietinum L.). *Int. J. Nat. Prod. Res*, *3*: 44-53.

41. Taye, G. M., Bule, M., Gadisa, D. A., Teka, F., & Abula, T. 2020. In vivo antidiabetic activity evaluation of aqueous and 80% methanolic extracts of leaves of *thymus schimperi* (Lamiaceae) in alloxan-induced diabetic mice. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *13*: 3205.

42. Althnaian, T., Albokhadaim, I., & El-Bahr, S. M. 2019. Hepatic gene expression, antioxidant enzymes and anti-diabetic effect of Nigella sativa in diabetic rats. *International Journal of Pharmacology*, *15*(2): 265-273.

43. Hamid, A., & Kalsoom, S. 2017. Comparative analysis of nutritional composition and effect of dietary fiber extracts of chickpea and Bengal gram on blood glucose and cholesterol levels of male induced diabetic and hypercholesterolemic rats. *Pakistan J. Zool*, *49*(2): 487-492.