Effect of Fractions of Alcoholic Extract of *Moringa oleifera* Lam. Bark on Dexamethasone induced Insulin Resistance in Rats

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ABSTRACT

Objective: Alcoholic extract of the bark of *Moringa oleifera* Lam. (MO), (Moringaceae), has been experimentally evaluated previously for its insulin sensitizing potentials. In the quest to explore the possibility of the class of phytochemical(s) responsible for this experimental claim, the alcoholic extract was fractionated and evaluated for insulin sensitizing effect in rat model for insulin resistance. **Methods:** Alcoholic extract of MO was fractionated into, non-polar [petroleum ether (PEF)], moderately non-polar [ethyl acetate (EAF)] and polar [aqueous (AQF)] fractions. The fractions obtained were investigated for their insulin sensitizing properties in dexamethasone induced insulin resistance in rats. The bioactive fraction was analysed by spectroscopy for further characterization of phytochemical(s) present. **Results:** Acute treatment for 4 h with dexamethasone (1 mg/kg i.p.) in rats led to the development of impaired oral glucose tolerance. Treatment with pioglitazone and EAF abolished dexamethasone induced oral glucose intolerance (OGT). Dexamethasone (1 mg/kg s.c., once daily for 11 d) administration led to the development of insulin resistance, characterised by fasting hyperglycemia, hyperinsulinemia, hypertriglyceri-demia, impaired OGT and increased HOMA IR index. Treatments with EAF (140 mg/kg p.o.) and pioglitazone [PIO (10 mg/kg p.o.)] significantly prevented dexamethasone induced metabolic changes. Similarly, treatment with AQF (95 mg/kg p.o.) also significantly prevented metabolic changes due to dexamethasone except impaired OGT. In contrast PEF (15 mg/kg p.o.) failed to prevent these metabolic changes except hypertriglyceridemia. **Conclusion:** The present study reveals that triterpenoid and the polyphenols (procyanidin) class of phytochemicals detected in EAF of alcoholic extract of MO bark may be responsible for the prevention of dexamethasone-induced insulin resistance in rats.

Key words: Dexamethasone, Insulin resistance, *Moringa oleifera*, Oral glucose tolerance test, Serum triglyceride.
Key message: This is the first report which confirms and speculates the presence of polyphenols and triterpenoids respectively in EAF of MO which may be responsible for preventing the development of insulin resistance in rat model.

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INTRODUCTION

Diabetes, fourth leading causes of death worldwide is a cluster of hyperinsulinemia, dyslipidemia, glucose intolerance, obesity and hypertension.[1] Insulin resistance, a prediabetic state of resistance towards the effects of insulin on its target tissues mainly in muscle, liver, and adipose tissue, is a central pathogenic feature of diabetes.[2] Currently, PPARγ agonists are used as insulin sensitizers in the treatment of diabetes.[3] Though, there are various techniques to manage diabetes and its complications, but still herbal formulations are the priority due to low treatment cost and lesser side effects. Around 80% of the world's population depends on traditional medicines derived from plants. Among the 21,000 medicinal plants used, 800 of them have been reported to be used in the treatment of diabetes worldwide.[4] Phytochemicals reported to have antidiabetic effect from these medicinal plants include alkaloids, triterpenoids, steroids, flavonoids, phenolics, iridoids, guanidines etc., and these phytochemicals may also have potential beneficial effects on insulin resistance which may be investigated. Given that insulin resistance increases the risk of type 2 diabetes and cardiovascular diseases, modulatory effect on insulin resistance may be the important mechanism through which these phytoconstituents can offer protection. Hence identification of potentially effective class of phytochemicals for insulin resistance state represents an attractive and potentially effective approach to the problem.

Moringa oleifera Lam., (MO) syn., *Moringa ptreygosperma* Gaertn., (Family-Moringaceae) commonly known as horseradish tree or drumstick tree.[5] All parts of this plant are being employed for the treatment of various ailments in the indigenous system of medicine. Traditionally the bark is used as cardiac and circulatory stimulant.[6] Experimental studies revealed, that the bark possess hypoglycaemic, antioxidant, cardioprotective effect and in our previous study we have reported the insulin sensitizing effect of the alcoholic extract of the bark.[7] Various class of phytochemicals are reported in the bark which includes alkaloids (moringine and moringinine),[8] glycosides (glucosinolate namely 4-(alpha-l-rhamnopyranosyloxy)-benzylglucosinolate),[9] tannins, triterpenoids,[10] and polyphenols (procyanidins).[11] In order to explore the possibility of insulin sensitization by any of the reported class of phytochemical(s) present in the alcoholic extract of the bark,[7] the present study was designed to test the various fractions of alcoholic extract in dexamethasone-induced chronic and acute rat model for insulin resistance.

MATERIALS AND METHODS

Chemicals

Dexamethasone (Dexona) injection was purchased from Zydus Cadila, Ahmedabad, India. Pioglitazone maleate was obtained as a gift sample from Torrent Pharmaceutical Ltd, Ahmedabad, India. Plasma biochemical estimation kits like glucose, triglyceride and total cholesterol were purchased

form Erba Transasia Bio-Medicals Ltd, Baddi, Himachal Pradesh, India. Plasma insulin estimation kit was purchased form MP Biomedicals, LLC, Eschwege, Germany, (kit code no. CT Insulin, lot no. INK 1223) Instrument used was Gamma counter, ECIL.

Plant material

The fresh bark of MO was collected in the month of November & December, from the local areas of Hubli–Dharward, Karnataka, India. The bark was authenticated by botanist Dr.Ganesh R. Hegde, Professor, P.G. Department of Botany, Karnatak University, Dharward, Karnataka, India and a voucher specimen bearing no. DOUN09017 is maintained in the herbarium of department of pharmacognosy of KLES College of pharmacy, Hubli, Karnataka, India.

Preparation of extract and fractions

The collected fresh bark of MO was dried and powdered coarsely. The bark coarse powder of 250 g was macerated at room temperature with 3×1 L ethanol 80 %v/v. Following filtration, the combined alcoholic extract was concentrated in-vacuo below 40 °C and finally dried in desiccator.

The crude alcoholic extract was fractionated into non-polar [petroleum ether (PEF)], moderately non-polar [ethyl acetate (EAF)] and polar [aqueous (AQF)] fractions by reconstituting 10 g in 1000 ml water and the solution was fractionated successively in a separating funnel with petroleum ether (60-80) (3×500 ml) and ethyl acetate (3×500 ml) followed by evaporation of organic solvent layers. Finally, the remaining aqueous layer was evaporated to dryness under reduced pressure.

Preliminary phytochemical investigation[12]

The fractions obtained were tested by qualitative chemical tests for the presence of phytoconstituents.

HPTLC Analysis

HPTLC analysis of the fractions were performed using precoated (silica gel 60 F_{254}; 0.25 mm thickness; size 20 x 20 cm; Merk, Darmstadt, Germany) TLC plates. Samples were prepared in methanol (10mg/ml) and were applied on the TLC plate by a Desaga auto sampler (AS 30 Desaga, Sarstedt Gruppe, Germany). The plates were developed in a CAMAG twin trough developing chamber (CAMAG, Muttenz, Switzerland) using chloroform and methanol (85:15) as mobile phase followed by air-drying and spraying with 5 % ethanolic phosphomolybdic acid,[10] 10 % ferric chloride and Liebermann-Burchard as detecting reagents on different plates respectively, which were developed similarly followed by heating the plates at 115 ºC for 5 min**.** The R_f values were determined by scanning the plates with densitometer (CD 60 Desaga, Sarstedt Gruppe, Germany) at 254nm, 366nm (before derivatization) and at 580 nm (after derivatization) using ProQuant 1.06 version software.

Animal Experiment

Male Wistar rats initially weighing 180 – 200 g were procured from the animal house of KLES College of pharmacy, Hubli, Karnataka, India. The rats were housed for 1 wk for acclimatization at controlled room temperature (22 ± 2 °C), natural 12h light: 12h dark cycle, free access to standard laboratory chow and water. After 1 wk of acclimatization, rats were randomly selected for different experimental groups. All the experimental procedures were carried out in accordance with committee for the purpose of control and supervision of experiments on animal (CPCSEA) guidelines. All the experimental protocols were reviewed and approved by Institutional Animal Ethical committee of KLES College of Pharmacy, Hubli, Karnataka, India.

Dose preparation of dexamethasone and fractions of MO

Dexamethasone (dexa) stock solution was prepared freshly in normal saline.[13,14] All the fractions of alcoholic extract of MO bark and pioglitazone were separately suspended in vehicle (1 % w/v Tween 80) to obtain the desired concentration and were administered orally.

Experimental design
Acute study

Twelve hours fasted rats were randomly divided into the six groups of six animals (n=6) each and received the following treatment, Group I (Control): received gavage of vehicle 1 ml/rat and normal saline 0.1ml/rat i.p., Group II (Dexa): received gavage of vehicle 1 ml/rat and treated with dexa1mg/kg i.p.,[13] Group III (PEF+Dexa): received gavage of PEF 15 mg/kg and dexa 1 mg/kg i.p., Group IV (EAF+Dexa): received gavage of EAF 140 mg/kg and dexa 1 mg/kg i.p., Group V (AQF+Dexa): received gavage of AQF 95 mg/kg and dexa 1 mg/kg i.p, Group VI (PIO+Dexa): received gavage of PIO 10 mg/kg and dexa 1 mg/kg i.p.[15]

Dexamethasone was administered 30 min after oral gavage of vehicle, fraction or PIO. After 4h of dexamethasone administration animals were subjected to OGTT.

Chronic Study

Rats were randomly divided into six groups of six animals (n=6) each and received the different treatments once daily for 11 d Group I (Control): received gavage of vehicle 1 ml/rat and normal saline 0.1 ml/rat s.c., Group II (Dexa): received gavage of vehicle 1 ml/rat and pre-standardized dose of dexa 1 mg/kg s.c.,[14] Group III (PEF+Dexa) received gavage of PEF 15 mg/kg and dexa 1 mg/kg s.c., Group IV (EAF+Dexa): received gavage of EAF 140 mg/kg and dexa 1 mg/kg s.c., Group V (AQF+Dexa): received gavage of AQF 95 mg/kg and dexa 1 mg/kg s.c., Group VI (PIO+Dexa): received gavage of PIO 10 mg/kg[15] and dexa 1 mg/kg s.c. Dexamethasone was administered 30 min after oral gavage of vehicle or fraction.

At the end of experimental period animals were fasted for 12 h and subjected to oral glucose tolerance test (OGTT). Blood samples were collected by retro-orbital plexus puncture under light ether anesthesia. Zero-hour blood samples were used for estimation of blood glucose, triglyceride, cholesterol and insulin. The samples collected were centrifuged at 3000 rpm for 20 min to separate out the serum which was then analysed.[7]

Oral glucose tolerance test [7]

On completion of study period oral glucose tolerance test (OGTT) was performed to measure the plasma glucose in each group. Glucose solution at a dose of 2 gm/kg (body weight) was administered by gavage. Blood samples were collected from the retro orbital plexus prior to glucose load (0 min) and post-glucose load at 30, 60, and 120 min.

Biochemical estimation

The concentration of plasma biochemical levels like glucose, triglyceride, total cholesterol and plasma insulin were measured by glucose oxidation method,[16] GPO–POD enzymatic method,[17] CHOD–POD enzymatic method[18] and radioimmuno assay method[19] respectively.

Assessments of Insulin Resistance and Sensitivity

The degree of insulin resistance was calculated by using Homeostasis Model Assessment (HOMA),[20]

$$HOMA = (fasting\ insulin \times fasting\ glucose)/405$$

Statistical analysis

All the results are expressed as mean standard error mean ($\pm$ S.E.M). Statistical analysis was carried out using Student's t-test and One-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons using GraphPad Prism 5 software. P values of less than 0.05 were regarded as significant (P<0.05).

Characterization of bioactive fraction

Fourier Transform Infrared (FT-IR) spectroscopy: A FT-IR spectrum was recorded on IR 200 Nicolet IR spectrophotometer (Thermo Electron Corporation, MA U.S.A.). Spectra were taken in KBr pellets.

LC-MS analysis: EAF 100 mg was dissolved in 10 ml of methanol and was sonicated for 10 min at 30°C in the ultrasonic bath. The sample was filtered through a 0.45-μm PVDF filter (Himedia labs. Pvt. Ltd., Mumbai, India) and was further used. Chromatography separation was performed using Surveyor Plus (Thermo Fisher Scientific, California, USA) HPLC system coupled to an MS analyser, LCQ Deca XP Max ion trap (Thermo finnigan, San Jose, CA, U.S.A.) with ESI interface using Xcalibur version 1.2 software for system control.

Sample was separated on 3 μm Dionex bonded silica C_{18} column (150 $\times$ 4.6 mm: Acclaim 120, Thermo Fisher Scientific, California, USA). A binary gradient elution was applied (solvent A, 0.1% formic acid in water and solvent B, methanol),[16] flow rate was set to 0.5 ml/min and injection volume was 10 μl.

All the MS-MS analysis was carried out in the negative mode $[M-H]^{-1}$ under the following optimized conditions: spray voltage, 4.49KV; capillary voltage, 19V; capillary temperature, 350°C; sheath gas (N_2), 65 arbitrary units; auxiliary gas (N_2), 10 arbitrary units; full scan spectra were recorded within the m/z range of 100-1000; Collision energy: 35%.

RESULTS

Preparation of extract and fractions

Three fractions were obtained viz., petroleum ether fraction (PEF) (yield 0.6 g, 6 %), ethyl acetate fraction (EAF) (yield 5.6 g, 56 %) and aqueous fraction (AQF) (yield 3.8g, 38 %).

Preliminary phytochemical investigation

Preliminary phytochemical studies exhibited the presence of steroids in PEF; triterpenoids and phenols in EAF; carbohydrates and glycosides in AQF respectively.

HPTLC Analysis

The HPTLC analysis of fractions revealed their finger print profiles. Petroleum ether fraction resolved 5 spots with Rf values 0.17, 0.20, 0.34, 0.56 and 0.67; ethyl acetate fraction resolved 7 spots with Rf values 0.25, 0.34, 0.41, 0.57, 0.65, 0.71 and 0.84; the aqueous fraction resolved 5 spots with Rf values 0.14, 0.39, 0.42, 0.54 and 0.59. Appearance of grey (with 10 % fecl3 reagent) or blue (LB reagent) spots was considered as positive evidence for the presence of phenols and triterpenes respectively[21] (Figure 1).

Animal Experiment
Acute study

Glucose intolerance developed after acute treatment of rats with dexamethasone (Control AUC 9923$\pm$441.3 vs Dexa AUC 12650$\pm$90.78, P<0.001) was significantly prevented in EAF treated (Dexa+EAF AUC 10284$\pm$239.3, P<0.001) and PIO treated (Dexa+PIO AUC 9845$\pm$311.1, P<0.001) rats. In contrast, PEF treatment further aggravated the dexamethasone induced glucose intolerance (Dexa+PEF AUC 15788$\pm$527.1, P<0.001). AQF treatment had no effect on this parameter (Figure 2).
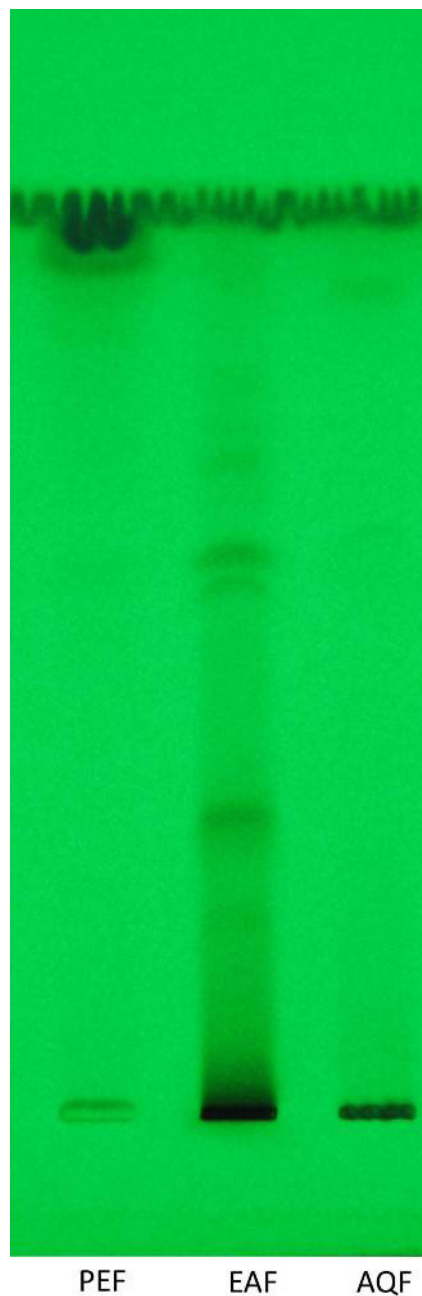

Figure 1: HPTLC chromatogram of PEF, EAF and AQF at UV 254nm.

Chronic study

As shown in Table 1, initial body weights of rats in all the groups were similar, dexamethasone treated rats lost the body weight significantly (P<0.001) compared to control rats. Treatment with PEF, EAF, AQF and PIO did not prevent the loss in body weight due to dexamethasone. Administration of dexamethasone showed significant increase in levels of fasting glucose (P<0.01), triglyceride (P<0.001), insulin (P<0.01) and HOMA IR (P<0.001). Treatment with EAF, AQF and PIO significantly (P<0.001) reduced fasting hyperglycemia, hypertriglyceridemia, hyperinsulinemia, HOMA IR and fasting hyperglycemia (EAF P<0.05; AQF P<0.05 and PIO P<0.001) induced by dexamethasone, whereas, treatment with PEF did not alter these metabolic changes. Dexamethasone, all the fractions and PIO tested did not show any effect on total cholesterol levels.

Table 1: Effect of PEF, EAF, AQF and PIO on body weight, triglyceride, cholesterol, glucose and insulin levels in dexamethasone treated rats (Chronic study).

Treatment groups	Body Weight		Triglyceride mg/dl	Cholesterol mg/dl	Glucose mg/dl	Insulin µU/ml	HOMA
	Initial	Final					
Control	190.0±3.000	208.0±4.066	56.14±2.122	64.85±1.327	89.18±5.130	52.83±4.277	11.82±1.539
Dexa	198.7±0.843	157.2[c]±2.725	297.8[c]±12.29	53.34±9.602	109.6[b]±0.680	78.00[b]±3.890	21.11[c]±1.076
Dexa +PEF	189.2±2.442	148.0±4.726	89.83[z]±2.748	39.65±2.782	100.0±9.148	63.50±5.233	15.84±2.199
Dexa +EAF	189.0±3.688	152.0±4.066	74.98[z]±5.596	39.61±3.586	92.83[x]±7.441	42.67[z]±6.020	10.05[z]±1.897
Dexa+ AQF	196.7±1.256	164.2±5.121	114.2[z]±12.92	49.37±3.574	92.11[x]±4.688	43.00[z]±2.944	9.740[z]±0.716
Dexa +PIO	196.7±1.202	152.0±2.955	70.62[z]±3.433	40.16±3.448	89.79[z]±2.909	40.33[z]±3.007	8.89[z]±0.727

Values are means ± SEM; Dexamethasone 1 mg/kg (Dexa); Dexamethasone+petroleum ether fraction 15 mg/kg (Dexa+PEF), Dexamethasone+ethyl acetate fraction 140 mg/kg (Dexa+EAF), Dexamethasone+aqueous fraction 95 mg/kg (Dexa+AQF), Dexamethasone+pioglitazone 10 mg/kg (Dexa+PIO).

[b]P< 0.01, [c]P< 0.001 Compared with Control rats.

[x]P< 0.05, [z]P< 0.001 Compared with Dexamethasone rats.
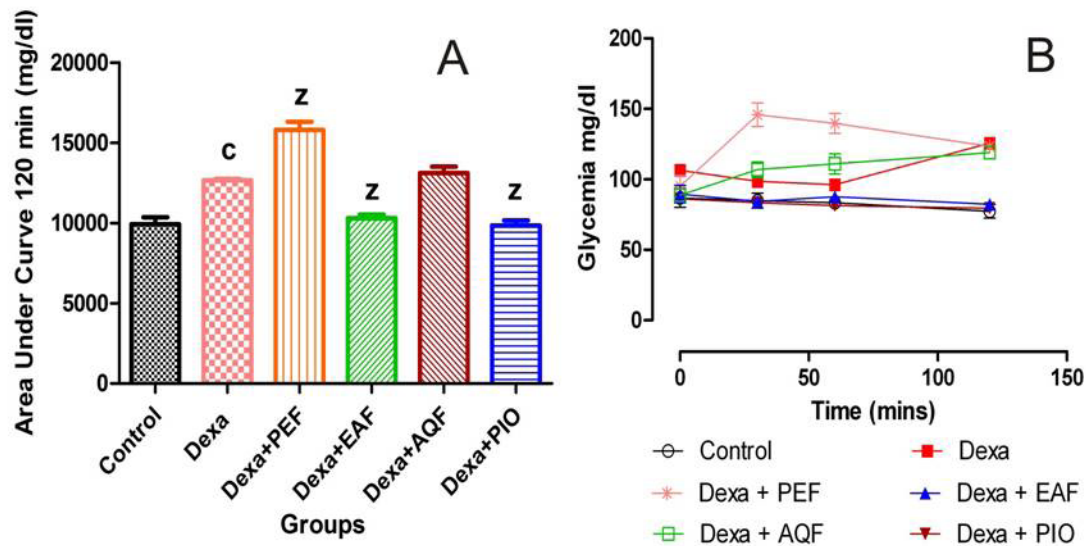
Values are means ± SEM; Area under curve (AUC); Dexamethasone 1 mg/kg (Dexa); Dexamethasone+Petroleum ether fraction 15 mg/kg (Dexa+PEF), Dexamethasone+Ethyl acetate fraction 140 mg/kg (Dexa+EAF), Dexamethasone+Aqueous fraction 95 mg/kg (Dexa+AQF), Dexamethasone+pioglitazone 10 mg/kg (Dexa+PIO).

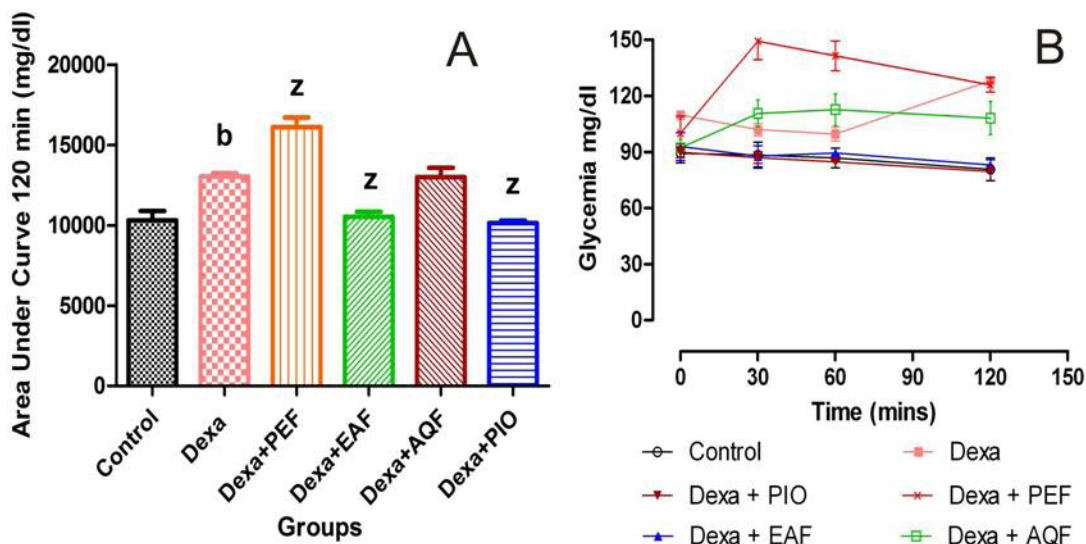[c]P< 0.001 vs Control rats.

[z]P< 0.001 vs Dexamethasone rats.

Figure 2: (A) Total area under curve of OGTT values from zero to 120 min, from control, Dexa, Dexa+PEF, Dexa+EAF, Dexa+AQF, Dexa+PIO groups and (B) Glycemia levels after oral glucose charge in tolerance test (Acute study).

Administration of dexamethasone led to the development of impaired glucose tolerance as indicated by significant increase in the sum of glucose levels during the OGTT in this group compared to control (Control AUC 10310±590.2 vs Dexa AUC 13020 ±222.6, P<0.01). Treatment with PIO and EAF prevented dexamethasone-induced impaired glucose tolerance as indicated by significant reduction in sum of glucose levels during OGTT compared to dexamethasone group (Dexa+PIO AUC 10149 ± 139.7, P<0.001; Dexa+EAF AUC 10529±314.5, P<0.001). Treatment with AQF had no effect on dexamethasone-induced glucose intolerance (Dexa+AQF AUC 12997±586.3). Further, treatment with PEF augmented significantly the effect of dexamethasone on the rise in sum of glucose levels during OGTT compared to dexamethasone group (Dexa+PEF AUC 16108±597.3, P<0.001) (Figure 3).

Characterization of bioactive fraction

FT-IR spectroscopy was used to augment the findings of preliminary phytochemical investigations of bioactive EAF. The EAF spectra showed absorption at 3354.90 (O-H stretching 3400-3100),[22] 2922.99 (asymmetric -C-H stretching 3000-2900),[23] 1613.86 (-C=C- aromatic stretching),[22] 1510.09 (-C=C-)[23], 1266.01(C-O stretching 1300-1000),[23] 807.24 and

Values are means ± SEM; Area under curve (AUC); Dexamethasone 1 mg/kg (Dexa); Dexamethasone+Petroleum ether fraction 15 mg/kg (Dexa+PEF), Dexamethasone+Ethyl acetate fraction 140 mg/kg (Dexa+EAF), Dexamethasone+Aqueous fraction 95 mg/kg (Dexa+AQF), Dexamethasone+pioglitazone 10 mg/kg (Dexa+PIO).
[b] $P< 0.01$ vs Control rats.
[z] $P< 0.001$ vs Dexamethasone rats.

Figure 3: (A) Total area under curve of OGTT values from zero to 120 min, from control, Dexa, Dexa+PEF, Dexa+EAF, Dexa+AQF, Dexa+PIO groups and (B) Glycemia levels after oral glucose charge in tolerance test (Chronic study).
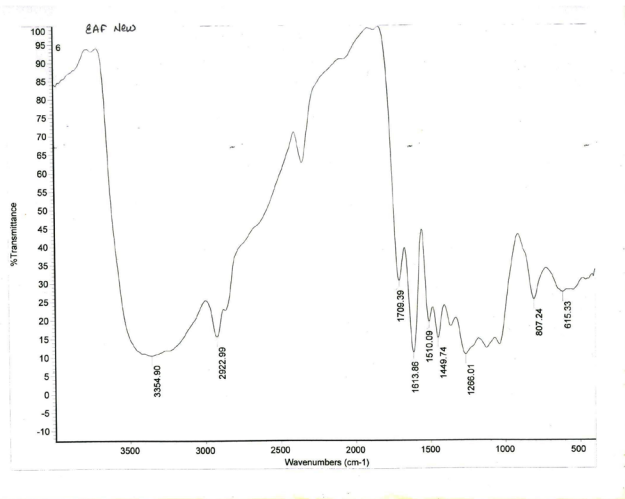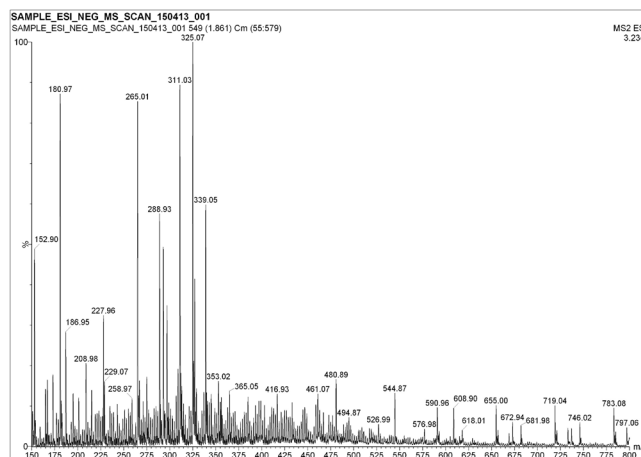

Figure 4: FT IR Spectrum of EAF.

Figure 5: LC MS Spectrum of EAF.

615.33 which are characteristic for polyphenol (procyanidin type).[22] The other absorptions at 1449.74(C-H bending cyclic (CH2)$_n$)[23] and 1709.39 (C=O characteristic for esters 1710-1690)[23] may be speculated due to the presence of triterpenes (Figure 4).

The LC MS and MS/MS (negative ion mode) analysis of bioactive EAF reveals that, some of the fragmentation values like m/z 576.98 and m/z 288.93 are in accordance with the reported fragmentation values[24] for deprotonated molecule of (epi)catechin dimer and (epi)catechin monomer

of a polyphenol (procyanidin type)[11] indicating its presence as one of the phytochemical in the ethyl acetate fraction (Figure 5).

DISCUSSION

The results of the current study demonstrate that EAF of MO prevents whole body IR, whereas, AQF of MO prevents hepatic IR induced by dexamethasone.

Our previous study has demonstrated that alcoholic extract of MO bark prevented IR at the dose of 250mg/kg.[7] The percentage yield of PEF, EAF

and AQF obtained from alcoholic extract in the present study were 6 %, 56 % and 38 % respectively. Based on these yield a quantity proportional to 250 mg was calculated for each fraction and employed as test dose.

At the tissue level IR is observed in skeletal muscle, adipose tissue and liver. Skeletal muscles are generally considered as major peripheral tissue responsible for disposal of blood glucose and impaired glucose tolerance is characteristic of peripheral IR whereas hepatic IR is generally characterized by fasting hyperglycemia and hyperinsulinemia.[25] Further, during IR antilipolytic effects of insulin is reduced which eventually leads to increased synthesis of TG from live.[26] In the present study, rats treated for 11 days developed whole body IR characterized by fasting hyperglycemia, hyperinsulinemia, hypertriglyceridemia and impaired oral glucose tolerance. These results are consistent with previous report.[27] In these rats EAF was effective in preventing both impaired oral glucose tolerance and fasting biochemical changes of IR, whereas, AQF was effective in abolishing only fasting biochemical changes of IR. These observations suggest that EAF was able to prevent dexamethasone induced IR in all target tissues of insulin, whereas, AQF was effective only at hepatic tissue.

By employing euglycemic- hyperinsulinemia clamp study in rats it is reported that single dose of daxamethasone induces IR.[14] Consistent with this report in the present study rats treated with single dose of dexamethasone developed IR as indicated by impaired oral glucose tolerance observed in these rats. Using this, model we tested the acute effect of different fractions on dexamethasone induced altered glucose uptake by peripheral tissues. Interestingly, only EAF could abolish dexamethasone induced impaired oral glucose tolerance, whereas PEF and AQF failed to modify this effect. These findings further support our observations made in chronic study that, EAF but not AQF of MO is effective in overcoming peripheral IR due to dexamethasone.

Qualitative, chemical identification tests and HPTLC analysis revealed the presence of polyphenols and triterpenoids in EAF. Further characterization of the bioactive EAF by FT-IR and LC MSn, analysis confirmed the presence of polyphenols (procyanidin) as one of the phytochemical present in the EAF on comparison with the previous literature values. Polyphenols and triterpenoids are some of the important phytochemicals reported to increase insulin sensitivity in different models of IR which supports our findings in this study.[28,29] Similarly, presence of glycosides in AQF may be responsible for abolishing IR in hepatic tissue observed in present study, since glycosides are reported to possess insulin sensitizing effects.[30]

CONCLUSION

This is the first report which confirms and speculates the presence of polyphenols and triterpenoids respectively in EAF of MO which may be responsible for preventing the development of IR in rat model. Isolation and pharmacological evaluation of phytoconstituents form these fractions may further confirm our finding.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

Authors do not have any conflict of interest.

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