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In search of suitable extraction technique for large scale commercial production of bioactive fraction for the treatment of diabetes: The case *Diospyros melanoxylon* Roxb.

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ABSTRACT

Diospyros melanoxylon Roxb. (*D. melanoxylon*) belongs to the family Ebenaceae and its leaves are very well known for making beedi throughout the World. The current study estimated the comparative extraction technique and its *in-vitro* antidiabetic prospective of the leaves of *D. melanoxylon*. Qualitative phytochemicals analysis of the samples from *D. melanoxylon* was carried out for the detection of secondary metabolites. Total phenolics, flavonoids, triterpenoids and tannins content of *D. melanoxylon* were estimated using colorimetric assay. Microwave-assisted extraction (MAE) technique with a low carbon output was observed for the speedy extraction of bioactive compounds obtained from *Diospyros melanoxylon* leaf extract. MAE produced a maximum yield of bioactive compounds which was found to be more efficient than ultrasound, soxhlet and maceration extraction. Qualitative HPLC analysis was performed for bioactive compounds. The *in-vitro* antidiabetic assay was performed using α-amylase and α-glucosidase inhibitory activity. In conclusion, the fractions exhibited the concentration-dependent inhibitory effect with significant ($P < 0.0001$) result. So the above performance might be accountable for the antidiabetic activity of *D. Melanoxylon* leaf extract due to presence of bioactive compounds.

1. Introduction

Diabetes Mellitus (DM) is a rising health trouble. Currently about 250 million people are living with diabetes and this figure is anticipated to be more than 366 million by 2030 according to WHO reports.[1] In calculation, nearly 3.2 million deaths per year are attributable to difficulties of diabetes; with six deaths every minute.[2] DM is a recurrent metabolic disorder which leads to the deficiency in the formation of insulin by the pancreas and has resulted significant morbidity and mortality because of microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular complications (heart attack, stroke and peripheral vascular disease) of patients.[3] DM is principally linked with carbohydrate, fat and protein metabolism which reduced production of insulin.[4] Treatments of insulin dependent diabetes or type 2 (T2DM) basically improves insulin sensitivity or reduces the rate of carbohydrate absorption from the gastrointestinal tract. Numerous side effects are observed for drugs which are used to treat T2DM, especially for those patients with liver and renal functional disorders.[5] Mammalian α-amylase is a prominent enzyme in the pancreatic juice, breaking down large and insoluble starch molecules into absorbable molecules, ultimately maltose.[6] On the other hand α-glucosidase is a membrane-bound enzyme at the epithelium of the small intestine and plays a key role in carbohydrate digestion. Inhibition of α-amylase and α-glucosidase leads to the delay or reduction of increased postprandial blood glucose levels. Thus, these two enzymes have been proposed as a potential therapeutic target for drug discovery in the treatment of T2DM.[7]

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Currently existing available drugs for the treatment of diabetes is not free from side effects. So, it is very important to identify and assess usually available natural drugs as alternatives to currently used antidiabetic drugs. In light of this fact, plants with proven ethnobotanical use can be the best option in developing natural products based alternatives.[8] Traditionally, a number of medicinal plants are used as antidiabetic and some of them have shown their efficacy through ethno-pharmacological evaluation. In addition, the World Health Organization also encourages to study and validate folklore claims for the treatment and prevention of diabetes and other lifestyle diseases which needs lifetime medication.[9,10]

In the present study the plant *D. melanoxylon* was selected for screening of antihyperglycemic activity on the basis of its ethnopharmacological information that the tribe of Chotta Nagpur region (Orissa) use it extensively as antidiabetic.[11] The traditional medicinal importance of the plant is ascertained as diuretic, carminative, laxative, styptic, good in epitaxis and night blindness, improves the eyesight, used in opthalmia, trichiasis, burns, tuberculosis glands, scabies, and old wounds.[12-14]

The preliminary phytochemical screening of *D. melanoxylon* showed the presence of steroids, triterpenoids in petroleum ether extract and flavonoids, tannins, phenolic compounds, sterols, triterpenoids in ethyl acetate extract.[15] Flavonoids, tannins, phenolic compounds, steroids in alcoholic extract and carbohydrates, proteins, amino acids, flavonoids, tannins, phenolic compounds, and tartaric acid as an organic acid in aqueous extract are also present.[16] The active phytoconstituents present in petroleum ether extract of *D. melanoxylon* were ceryl alcohol, lupeol, betulin and β-sitosterol.[17,18]

A large number of bioactive triterpenoids have shown multiple biological activities with apparent effects on glucose absorption, glucose uptake, insulin secretion, diabetic vascular dysfunction, retinopathy and nephropathy. The versatility of the pentacyclic triterpenes provides a promising approach for diabetes management and antiadipogenic activity.[19]

Basically polyphenolic compounds viz. Phenols and flavonoids were mainly attributed for the antidiabetic property.[20] Attempts have been made to determine their potential in preventing β-cell apoptosis, promoting β-cell proliferation and insulin secretion and enhancing insulin activity.[21] It is reported that the flavonoid compound rutin of *Ruta graveolens* is pharmacologically active and has the capability to control insulin activity and insulin resistance in type 2 diabetic rats.[22] It has been demonstrated that the flavonoids act as insulin secretagogues or insulin mimetics, probably by influencing the pleiotropic mechanisms of insulin signaling in diabetes mellitus.[23] The antidiabetic activity of triterpenoid saponin is thought to be due to reversing of atropy of the pancreatic islet of β-cells, as a result of which there may be increased insulin secretion and increase in the hepatic glycogen level and these may attenuate hyperinsulaenimia. The α-adrenergic blocking effect also might contribute to their insulin secretion and sensitizing effects.[24] *D. melanoxylon* is used in the management of diverse diseases and treatment of diabetes, anaemia, inflammation of spleen, dyspepsia, diarrhoea, scabies, hypotensive and used as carminative, laxative, diuretic and astringent.[15]

Choice of suitable extraction technique is very important for extract preparation. Traditional extraction methods need lengthy time and also are less effective. With conventional methods of extraction (such as Soxhlet) the fear of thermal degradation of bioactives cannot be ruled out.[25] Traditionally, for the extraction of triterpenoids, heat reflux and Soxhlet extraction techniques had been the first line of choice[26,27] but they are definitely not greener methods which are the need of the hour. Such methods require large volume of organic solvent and consume more electricity thus increasing the carbon load. In contrast, microwave-assisted extraction (MAE) is known for its better efficacy, good reproducibility, low consumption of organic solvents and time, and low carbon dioxide production. MAE is based on volumetric heating where the solvent and sample matrix is heated simultaneously in a volumetric fashion.[28] Several applications of MAE for biologically active compounds have appeared in the literatures, such as extraction of coumarin and related compounds from *Melilotus officinalis*,[29] extraction of tanshinones from *Salvia miltiorrhiza*,[30] extraction of flavonoids from *Radix astragali*,[31] and extraction of oleanolic acid from *Gymnema sylvestre*.[32]

The broad objective of this work is to develop a microwave based extraction protocol for the large scale production of bioactive fractions of *D. melanoxylon* leaves to be useful for the treatment of diabetes and compare its efficiency with that of conventionally used methods. This study was also undertaken to confirm that the said plant may be used as a future drug candidate for the treatment of diabetes through α-amylase and α-glucosidase enzyme inhibition.

2. Materials and methods

2.1. Plant collection

The leaves of *D. Melanoxylon* were collected in the month of April, 2014 from Dhenkanal District, Bhubaneswar, Odisha. The plant was authenticated from Botanical survey of India, Central National Herbarium, PO- Botanic Garden, Howrah-711103. The specimen voucher (Reference no. CNH/28/2014/Tech.II/HAR-03) was deposited in Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India. The leaves were washed properly, shade dried for 20 days and cut into small pieces. The dried leaves were ground using mechanical grinder, passed through sieve number 60 to obtain a homogeneous leaf powder and stored in an airtight container for experimental trial.

2.2. Apparatus and condition

The extraction system comprised of microwave extractor (CATA R) manufactured by Catalyst Systems (Pune, India) equipped with a magnetron of 2450 MHz with a maximum power of 700 W (100%), a reflux unit, 10 power levels (140 W (20%) to 700 W (100%)), time controller, temperature sensor, exhaust system, beam reflector and a stirring device. Ultrasound assistant extraction (UAE) was conducted with an ultrasound probe (Labsonic WM, Sartorious, Goettingen, Germany).

2.3. Extraction and fractionation

2.3.1. Soxhlet extraction

Exhaustive Soxhlet extraction was performed using a classical Soxhlet apparatus with accurately weighed 20 g sample of the drug powder screened through sieve number 60 for 25 h by hot percolation method using a hydro-methanolic mixture (95:05). After extraction, the extract was passed through Whatman no. 1 mm membrane filter, evaporated under reduced pressure and preserved for experimental trials.

2.3.2. Maceration extraction

Maceration was carried out in a closed conical flask of 250 ml for 5 days. 20 g powdered leaf sample was taken and extracted with 100 ml methanol: water (95:05). Occasionally shaking of the conical flask was carried out. Heat was not applied in either of the cases. The marc was separated by centrifugation (for15 min at 4 °C and 4000 rpm, R-8C, REMI, Mumbai, India) and the supernatant was then evaporated under reduced pressure and preserved for experimental analysis.

2.3.3. Ultrasound assisted extraction

20 g of powdered leaf sample was extracted with (5 g × 4 assembly) 100 ml (25 ml × 4 assembly) of the previously mentioned solvent. Extract was concentrated in the same manner as explained earlier.

2.3.4. Microwave assisted extraction

20 g leaf powdered sample was extracted with 150 ml extraction solvent as mentioned earlier. The microwave operating conditions were as follows, Power: 500 W, extraction time: 8 min, solvent: sample ratio loading- 30:4 ml/g and preleaching time: 10 min. Preleaching time is the contact time between sample matrix and extracting solvent before microwave irradiation. 10 min preleaching time is favourable for enhancing the extraction yield and allows sufficient swelling of the plant matrix. This increased hydrated status helps is bursting of the cell due to internal thermal stress and also extension of the cellular pores thus facilitating leaching of the target analyte.[32] After extraction, sample was concentrated as explained earlier. Methanol was used as the extracting solvent due to its better solubilising capacity for triterpenoids. A literature search revealed that methanol has been used frequently for the extraction of triterpenoids. Moreover, due to its better dissipation factor (tan d = 0.6400), methanol would heat up better in the MAE process.

2.3.5. Preparation of different fractions

All the extract viz. soxhlet extract, macerated extract, ultrasound extract, and microwave treated extract were dissolved one by one in double distilled water separately and passed through 1 mm membrane filter paper. Subsequently with the aqueous fraction (AQF) three more fractions such as n-hexane (NHF), chloroform fraction (CF) and ethyl acetate fraction (EAF) was prepared using liquid-liquid partitioning system with the help of a separating funnel. Mixing was completed by inverting the funnel 6 times (pressure in the separating funnel was discharged after each invert), where after the mixture was allowed to form two layers and both layers were collected separately. The process was repeated six times. The entire fractions were made solvent free by vacuum evaporation and refrigerated till further use (see Fig. 1).

2.4. Scanning electron micrographs

In order to understand the extraction mechanism, marc obtained from various extraction methods were subjected to scanning electron micrographs. After removing the solvent, the remaining *D. melanoxylon* leaf samples were plunged in liquid nitrogen and then cut with a cold knife. The sectioned particles were fixed on a specimen holder with aluminium tape and then sputtered with platinum. All the specimens were examined with a JEOL JSM-6700F (Akishima, Tokyo, Japan) scanning electron microscopy under high vacuum condition at different magnification.

2.5. Phytochemical analysis

Phytochemical analysis was carried out to identify secondary metabolites of *D. melanoxylon* fractions obtained from different extraction methods. The fractions were tested for alkaloids, glycosides, carbohydrates, terpenoids, flavonoids, saponins and tannins. Phytochemical analysis was performed based on previously reported methods with slight modifications.[33,34]

2.6. High performance liquid chromatography (HPLC) analysis

The various test fractions (1 mg) of each extraction method (Soxhlet extract, Macerated extract, ultrasound extract and microwave assisted extract) was dissolved in 2 ml methanol and was vortexed for 2 h for proper mixing. The final solution was filtered using 0.22 mm membrane filter (Biotech, Germany) prior to high performance liquid chromatography (HPLC) analysis.

The identification of compounds on HPLC (FRC-10A, UFLC, SHIMADZU, JAPAN) with PDA detector was performed using C18, 4.2 mm × 250 mm, 5 μm column. The chromatographic separation was carried out using solvent methanol (100%) and TFA 0.1% with 1 ml/min flow rate using UV–Vis detector at 280 nm wavelength at cool temperature at injection rate of 10 μl.

The identification of each compound was established by comparing the retention time and UV–Vis spectra of the peaks with those previously obtained by injection of pure external standard compounds (gallic acid, ellagic acid, ferulic acid, rutin and quercetin).

2.7. Determination of total polyphenol content

Total polyphenolic content (TPC) was performed by using the method of Koncić et al. (2010) with slight modification.[35] The method was performed in triplicate using Folin-Ciocalteu reagent to determine total phenolic content in different fraction of different method obtained from *D. melanoxylon*. The Folin-Ciocalteau assess depend on the basis of transmission of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes. Momentarily, 75 μl of diluted fraction and 425 μl of double distilled water were added to 500 μl Folin-Ciocalteu reagent and 500 μl of Na_2CO_3 (10% *w*/*v*). The combination was mixed and incubated for 45 min in the dark at 37 °C temperature. After incubation, the absorbance was measured at 765 nm using a UV–Vis spectrophotometer. Total phenol content was expressed in milligrams of Gallic acid equivalents per g of dried sample (mg GAE/g sample) using the expression from the calibration curve $Y = 0.0556x - 0.1909$, $R^2 = 0.9929$.

2.8. Determination of total flavonoid content

Total flavonoid content (TFC) was performed by the method of Russo et al. (2015) with slight modification.[36] Briefly, 150 μl of different fractions was added to 45 μl of 3% $NaNO_3$ into microcentrifuge tube. After 10 min, 90 μl of 2% $AlCl_3$ was added and at the 6th minute, 300 μl of 1 M NaOH solution was added and the total volume was made up to 1.5 ml with double distilled water. The mixture was mixed well and the absorbance was measured against reagent blank at 465 nm after 30 min of incubation at 45 °C temperature. Quercetin was used as standard drug to plot the calibration curve. Total flavonoid content was expressed in milligrams of Quercetin Equivalent per g of dried sample (mg QE/g sample) using the equation obtained from the calibration curve $Y = 0.0006x + 0.001$, $R^2 = 0.9953$.

2.9. Determination of total triterpenoid content

Total triterpenoid content (TTC) was determined by the method of Fan and He (2006) with slight modification.[37] 100 μl of different fractions (0.25–1.25 mg/ml) were mixed individually with 150 μl (7.5% w/v) vanillin-glacial acetic acid solutions and 500 μl of perchloric acid solution. The sample solutions were heated for 30 min at 60 °C and then cooled in an ice-water bath to ambient temperature. After the addition of 2.25 ml glacial acetic acid, each sample solution's absorbance was measured at 548 nm using a UV–visible spectrophotometer. Total triterpenoid content was expressed in milligrams of Lupeol equivalents per g of dried samples (mg LPE/g dry sample) using the equation obtained from the calibration curve $Y = 0.0143x + 0.1546$, $R^2 = 0.9947$.

2.10. Determination of total tannin content

Total tannin content (TTC) of various samples was evaluated according to the method of Russo et al. (2015) with slight modification.[36] 500 μl of bovine serum albumin (BSA) solution in 0.2 mol/l acetic buffer, pH 5.7 with 0.17 mol/l NaCl (1.5 mg/ml) was added to various concentrations of sample (0.25–1.25 mg/ml) and mixed carefully. After 20 min, the samples were centrifuged at 5000 g for 20 min. The supernatant was removed, and the pellet dissolved in 1 ml of1% aqueous solution of sodium dodecyl sulphate (SDS) and 4% triethanolamine. After that, 250 μl of 0.01 mol/l FeCl3 in 0.01 mol/l HCl was added. After 45 min the absorbance reading was taken at 510 nm. Total tannin content was expressed as mg of tannic acid equivalent/g of sample (mg TAE/g of sample) using the equation obtained from the calibration curve $Y = 0.046x + 0.0796$, $R^2 = 0.9974$.

2.11. α-amylase inhibitory activity

α-amylase inhibition assay was carried out using a microplate reader according to the method of Sudha et al. (2011) based on the starch-iodine test with little modification.[38] 75 μl of different concentrations of fractions (100–500 mg/ml) were added to 150 μl 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) and 75 μl α-amylase solution, and incubated at 37 °C for 15 min. Take 100 μl from each sample reaction solution, 750 μl soluble starch (1%, w/v) and 500 μl phosphate buffer solution was added and incubated at 37 °C for 45 min. Take 25 μl of above mixture and added 2.5 ml of iodine reagent (5 mM I_2 and 5 mM KI) and mixed properly for uniform mixture. The color change was observed and the absorbance was taken at 565 nm on a microplate reader. Absence of sample is the control reaction representing 100% enzymatic activity. To eliminate the absorbance produced by plant fractions, appropriate fraction controls without the enzyme were also included. The standard drug acarbose (α-amylase inhibitor) was used as a positive control. It is observed the dark-blue color which indicates the presence of starch, a brownish color indicates partially degraded starch and a yellow color indicates the absence of starch in the reaction mixture. In the presence of inhibitors from the fraction the starch added to the enzyme assay mixture is not degraded and gives a dark blue color complex whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolysed by α-amylase.

2.12. Inhibition of α-glucosidase activity

The α-glucosidase enzyme inhibition activity was carried out according to the method of kuppusamy et al. 2011.[39] It was determined by incubating100 μl of α-glucosidase enzyme (1 U/ml) solution with100 μl of phosphate buffer (pH 7.0) which contains 100 μl of enzyme inhibitor such as acarbose (3.6–50 μg/ml) at 37 °C for 60 min in maltose solution. To stop the α-glucosidase action on maltose, the above reaction mixture was kept in boiling water for 3 min and cooled. To this, 2 ml of glucose reagent was added and its absorbance was measured at 540 nm to measure the amount of liberated glucose by the action of α-glucosidase enzyme. The percentage inhibition and 50% inhibitory concentration (IC_{50}) value was calculated.

2.13. Statistical analysis, determination of percentage inhibition and IC_{50} value

In this study, data were expressed as mean ± standard deviation (SD). Statistical analysis was performed (Graph pad prism version 7.02) using two way analysis of variance (ANOVA) and significant interactions were assessed by Newman-Keuls post hoc test. The percentage inhibition of α-amylase and α-glucosidase was calculated using the following formula:

$$\%\ \text{inhibition} = (\text{Absorbance of control} - \text{Absorbance of test})/\text{Absorbance of control} \times 100.$$

3. Results and discussion

3.1. Effect of microwave power and irradiation time

Microwave power of 500 W with irradiation time 8 min was used for the extraction of *D. Melanoxylon* leaf sample. The above operating conditions were chosen based on the earlier reports of our research group.[32] MAE is directly correlated with effects of microwave energy on phytomolecules by ionic conduction and dipole rotation which result in power dissipated in a volumetric fashion inside the solvent and plant material and then generate molecular movement and heating.

3.2. Effect of preleaching time and solvent to material ratio

Preleaching time can be defined as the contact time between sample matrix and extracting solvent before microwave irradiation. Preleaching time of 10 min is favourable enough to allow sufficient swelling of the plant matrix for enhancing the extraction yield. This increased hydrated status helps is bursting of the cell due to internal thermal stress and also enlargement of the cellular pores thus facilitating leaching of the target analyte.[30]

The solvent volume always must be sufficient to ensure that the entire sample is immersed during the extraction process. Generally in conventional extraction techniques a higher volume of solvent will increase the extraction performance, but in MAE a higher solvent volume may give lower yield.[40,41] According to Mandal and Mandal 2010, before the ratio of solvent to material reached, at which the yield reached its highest value. And then it fell down slightly due to an inadequate stirring of the solvent when the microwaves are applied at larger volumes. Additionally, big volume of solvent cause more absorption of microwave energy and satisfactory microwave energy not available for helping the cell fracture for efficient leaching out of the intent analyte.[42,43]

3.3. Extraction mechanism

In order to study the extraction mechanism the marc of *D. melanoxylon* obtained after each extraction technique were examined by a scanning electron microscopy. Fig. 2A–H represent the micrographs of the maceration extraction sample, ultrasound treated sample, soxhlet extraction sample, and microwave treated sample respectively both at the surface and cellular levels. The changes observed for macerated sample were not intense and only few slight ruptures took place on the surface of the sample accompanied by slight widening of the cellular channels. Soxhlet extraction process revealed more ruptures has taken place on the surface as well as internally at the cellular level. On the other hand the surface of the sample was greatly destroyed after microwave treatment followed by massive widening or opening up of the cellular pores. This observation suggests that microwave treatment affects the structure of the cell due to the sudden temperature rise and the internal pressure increase. The higher temperature attained by the cell wall, during MAE, causes dehydration of

cellulose and reduces its mechanical strength, which allows the solvent to gain an easy entry inside the cellular channels.[44]

But, another interesting theory of synergism between mass transfer and heat transfer can also be put forward in support of the accelerated extraction due to microwave effect. Solid-liquid extraction may be thought as a phase transfer of solute from one phase to another. The transfer mechanism is governed by capillary flow and depends upon solvent viscosity. In conventional solvent extraction, mass transfer occurs from the inside to the outside while heat transfer occurs from the outside to the inside. For

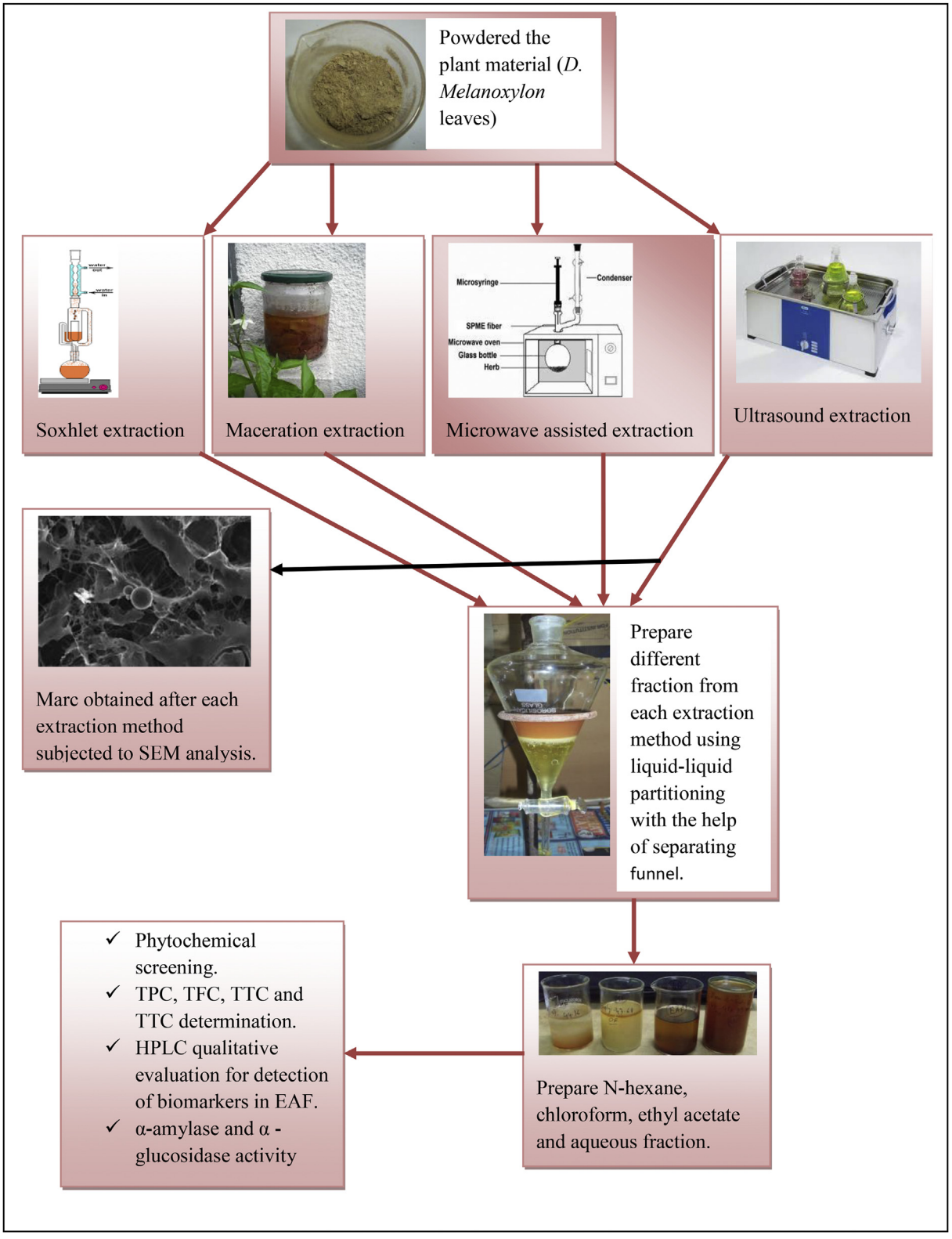

Fig. 1. Flowchart for fractionation and antidibetic activity performed of *D. melanoxylon* leaves.

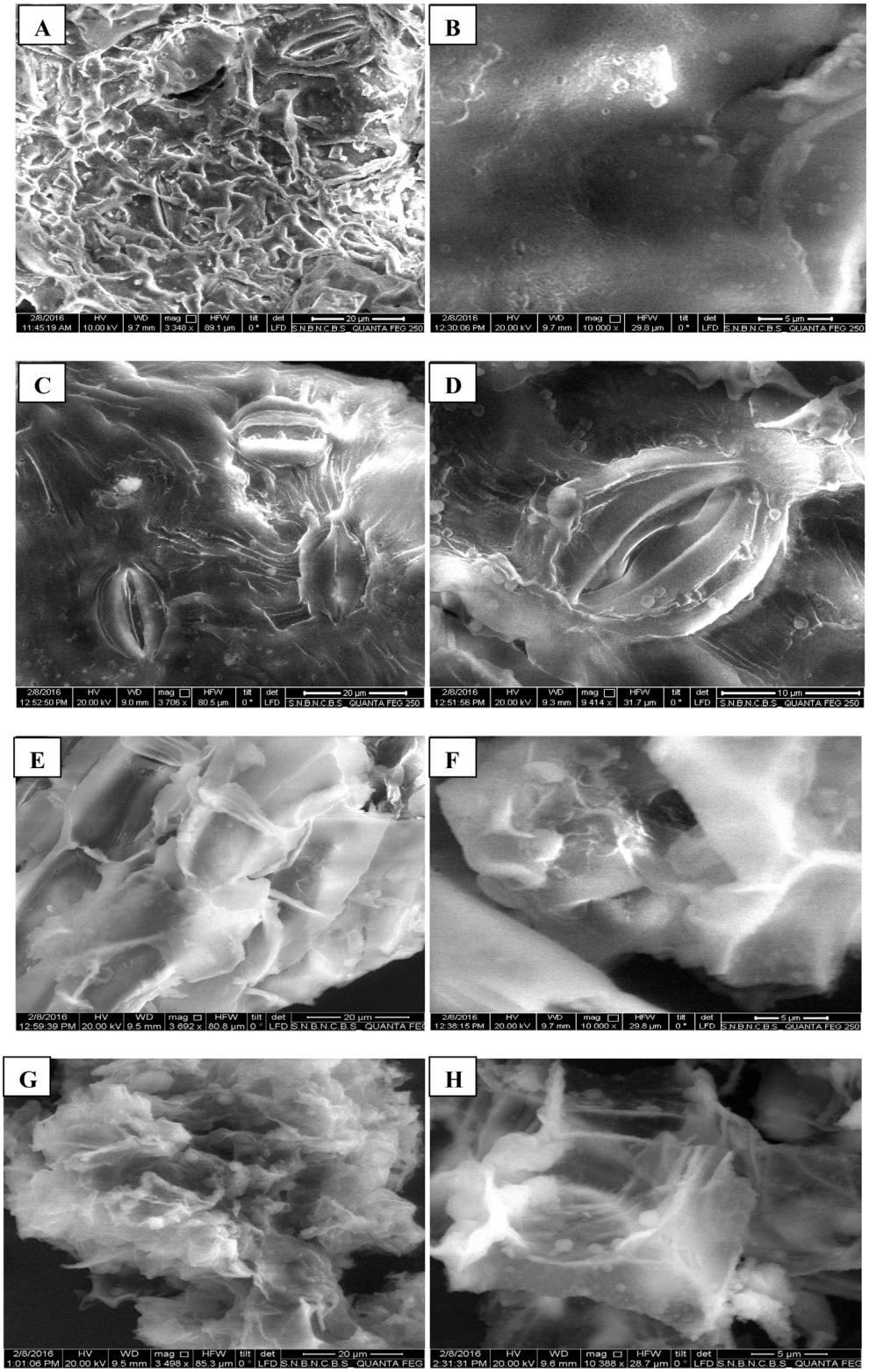

Fig. 2. Scanning electron micrographs of leaf sample. (A) Surface view, (B) cellular channels and internal pores of maceration sample. (C) Surface view, (D) cellular channels and internal pores of ultra sound leaf sample. (E) Surface view, (F) cellular channels and internal pores of soxhlet leaf sample. (G) Surface view, (H) cellular channels and internal pores of microwave assistant leaf sample.

microwave solvent extraction, due to improved hydration status of the plant cell, internal heating inside the plant cell takes place thus making the two transport phenomena work in the same direction from the inside of the extracted material to the bulk solvent. The acceleration of extraction rates under microwaves could be due to a synergy combination of the two transfer phenomena mass and heat acting in the same direction. In microwave solvent extraction, heat is dissipated volumetrically inside the irradiated medium, while in

conventional solvent extraction; heat is transferred from the heating medium to the interior of the sample.

3.4. Comparison of MAE with other conventional techniques

The selection of an extraction method would mainly depend on the advantages and disadvantages of the processes, such as extraction yield, complexity, production cost, environmental friendliness, pharmacological activity and safety. MAE is a relatively new method, which has received increasing attention as an alternative green method. The principle of heating during MAE is based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation. Ionic conduction is the electrophoretic migration of ions when an electromagnetic field is applied. The resistance of the solution to this flow of ions will result in friction and therefore heat the solution.

Dipole rotation means realignment with the applied field. At 2.45 GHz, which is the frequency used in commercial systems, the dipoles align, randomize and jostle 4.9 × 109 times per second and this results in heating.[45] In the current study, MAE was compared with the other conventional extraction techniques for the extraction of *D. melanoxylon*. The conditions of different techniques and their results are summarized in Table 1 and showed that in terms of yield of target analyte. The best results were obtained by MAE, which gave significantly more. On extraction time, MAE was also the fastest extraction method with only 8 min of extraction time and preleaching time of 10 min. Maceration and soxhlet extractions are time consuming processes based on heat or mixing to increase the mass transfer rate. Ultrasound extraction is not so time consuming but not so effective method. MAE was found to be 85.47%, 32.11% and 214.68% more effective when compared to ultrasound extraction, soxhlet extraction and maceration extraction, respectively. These features along with an ease of operation and implementation would position MAE as a valuable and cost effective technology suitable for today's highly competitive industries with growing demand for increased productivity, improved efficiency and reduced cycle time.

3.5. Phytochemical compositions

The fractions of *D. melanoxylon* leaf obtained from this study contained carbohydrates, terpenoids, flavonoids, saponins and tannins but not alkaloids and steroid.

3.6. High performance liquid chromatography (HPLC) analysis

EAF from various extraction methods obtained from *D. Melanoxylon* leaves was analyzed for identification of polyphenolic (phenolic acids and flavonoids) compounds by HPLC-DAD analysis. We have determined the HPLC analysis of only EAF as our previous work proved that this fraction is more active. Typical chromatograms of EAF and standards are presented in Fig. 3. Compounds were identified by comparison of retention times with standard reference compounds viz., four phenolic compounds (gallic acid, ellagic acid, ferulic acid, and rutin) and one flavonoid (quercetin).

Phenolic and flavonoid derivatives were detected in the HPLC-DAD profiles of the four samples. In the group of phenolic acids, gallic acid was the major compound detected in all the samples with retention time 12.277 min. Basically in MAE-EAF content higher concentration of gallic acid. Ferulic acid was the minor compound detected in all the samples with retention time 23.017 min. Then rutin was the second major compound in all the samples with retention time 24.708 min. Ellagic acid was present in the entire sample with retention time 25.454 min. Ellagic acid content is more in MAE-EAF comparatively. Minor concentration of quercetin compound was present to the entire fractions with retention time 28.08 min. Among the different extraction method of *D. Melanoxylon* leaf, MAE-EAF was exposed as an effective solvent for extraction of most of phenolic acids and flavonoid.

3.7. Total polyphenolic and flavonoid content

Total polyphenolic and flavonoid content was analyzed systematically. Results are shown in Table 2. The total polyphenolic content is expressed as gallic acid equivalent (mg GAE/g dry sample) and results were calculated from the standard gallic acid calibration curve ($R^2 = 0.992$). The total flavonoid content is expressed in Quercetin equivalents (mg QE/g dry sample) and results were calculated from the standard Quercetin calibration curve ($R^2 = 0.995$). The results are also in agreement with the findings of HPLC analysis.

3.8. Total triterpenoid and tannin content

The total triterpenoid content is expressed in milligrams of Lupeol equivalents per gram of dried samples (mg LPE/g dry sample) and results were calculated from the standard Lupeol calibration curve ($R^2 = 0.994$). Tannin content was expressed as mg tannic acid equivalent (TAE)/g of dried sample by using a standard curve ($R^2 = 0.9974$) and results are reported in Table 2.

3.9. In vitro antidiabetic activity

Drugs that inhibit hyperglycaemia by suppressing hydrolysis of starch for α-amylase and carbohydrate for α-glucosidase have been extensively used in the management of diabetes mellitus.[28,29] Many herbal extracts have been reported for their anti-diabetic activities and are currently being used in Ayurveda for the treatment of diabetes. However, such medicinal plants have not gained much importance as medicines due to the lack of sustained scientific evidence.

In the present study, different fractions from various extraction methods of *D. Melanoxylon* leaves were screened for their α-amylase and α-glucosidase inhibitory activity. Several studies performed on this plant state them to be hypoglycemic, but no reports exist on study for pancreatic α-amylase inhibitors in order to justify their hypoglycemic property. The rationale for performing extractions from non-polar to polar solvents is to confirm and validate the inhibitory activity in the aqueous extractions performed in the traditional manner as well as to search for newer, more potent inhibitory compounds in the organic solvents. Primary screening

Table 1
Comparison of MAE with other conventional techniques (extraction time, solvent volume, percentage of yield, and solvent ratio).

Extraction method	Extraction time	Solvent Volume (ml)	Percentage of yield	Solvent ratio
Soxhlet	16 h	200	17.25	Methanol: Water (95:05 v/v)
Maceration	4 days	200	7.22	
USE	20 min	150	12.25	
MAE	8 min	100	22.72	

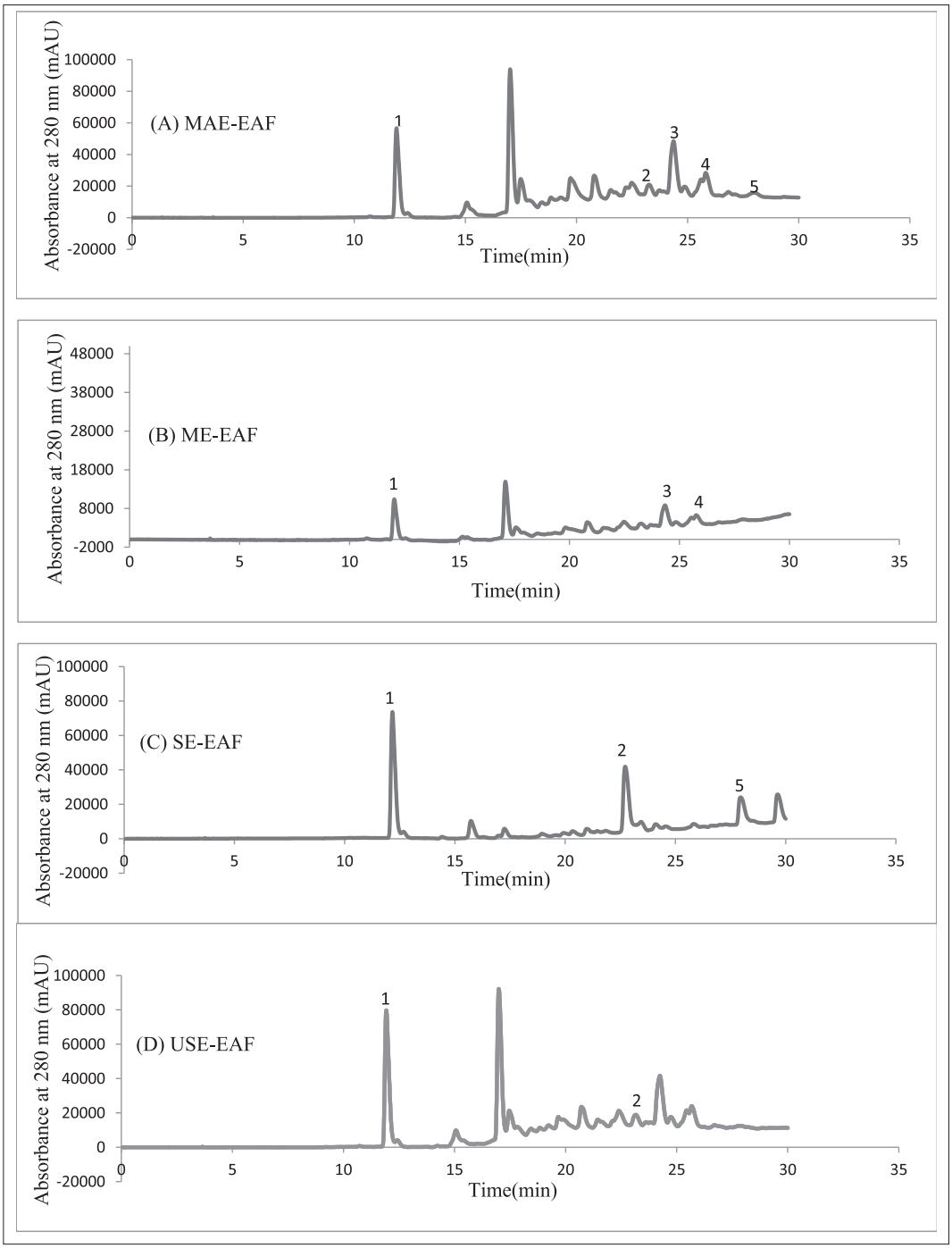

Fig. 3. Identification of the major polyphenolic compounds in chromatograms of Ethyl acetate fraction (EAF) of (A) MAE-EAF = Microwave assistant extract, (B) ME-EAF = Maceration extract, (C) SE-EAF = Soxhlet extract, (D) USE-EAF = Ultrasound extract. Standard compounds (E) GA = Gallic acid, (F) Ferulic acid, (G) R = Rutin, (H) EA = Ellagic acid, and (I) QE = Quercetin. Peak 1 = Gallic acid; 2 = Ferulic acid; 3 = Rutin; 4 = Ellagic acid; 5 = Quercetin.

for α-amylase inhibition was performed based on starch-iodine color complex formation. On the other hand α-glucosidase is a membrane-bound enzyme at the epithelium of the small intestine and plays a key role in carbohydrate digestion.

Catherine et al. have reported the antidiabetic effect (α-amylase and α-glucosidase inhibition activities) of tannins and results have revealed cheering effects.[46] Flavonoids have also been demonstrated to suppress glucose level considerably as a strong inhibitor of diabetes.[47] Koneri *et al.* reported that the triterpenoid saponin of *M. Cymbalaria* possesses potential antidiabetic activity which may be endorsed to modulation of calcium channel and β-cell rejuvenation. So a number of scientific reports indicate phenolics, flavonoids, triterpenoids and tannins have antidiabetic role through their effects on different possible mechanism.[48]

Amongst the entire fraction, ethyl acetate fraction from soxhlet extract (IC_{50} 67.30 ± 0.75 μg/ml), maceration extract (IC_{50} 94.52 ± 0.85 μg/ml), ultrasound extract (IC_{50} 74.27 ± 0.89 μg/ml) and microwave extract (IC_{50} 52.39 ± 1.21 μg/ml) have shown better

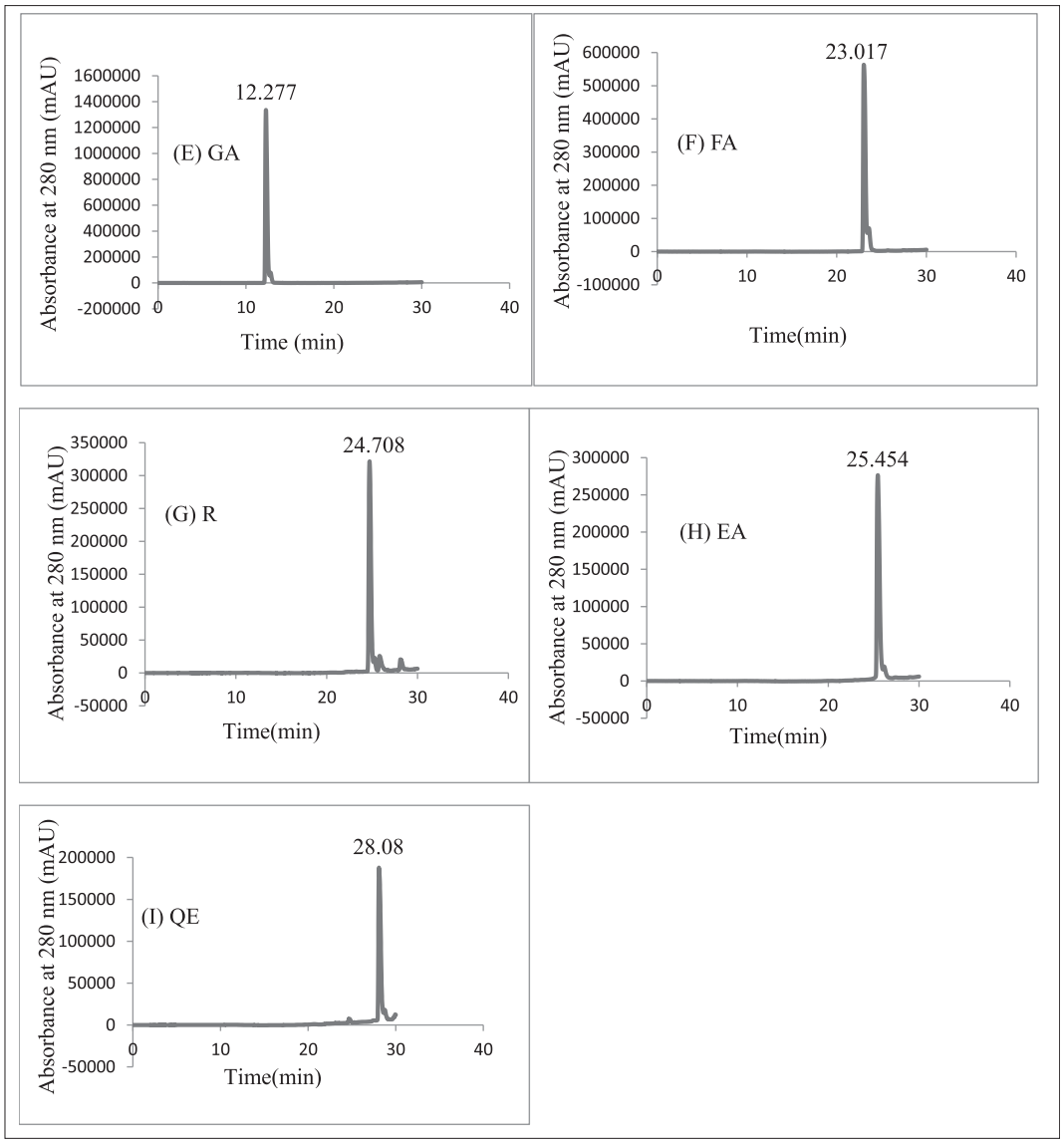

Fig. 3. (*continued*).

α-amylase enzyme inhibitory activity.

Acarbose was used as standard drug with an IC_{50} value of 72.22 ± 0.94 of α-amylase and 87.36 ± 0.86 of α-glucosidase for both inhibition assays and the standard curves are shown in Fig. 5. Data obtained (Table 3) reveal that ethyl acetate fraction obtained from microwave extraction showed significantly better inhibiton of α-amylase than α-glucosidase, as presented in Figs. 4 and 5. Since the ethyl acetate fraction (EAF) was found to be more active, so using Post hoc test the EAF fraction obtained from all extraction methods was compared with each other and also with acarbose. Results of Post hoc test clearly indicates that EAF obtained from Soxhlet and ultrasound method produced similar results to that of acarbose (standard drug) in terms of α-amylase inhibitory activity, whereas EAF extract produced from MAE showed significantly ($p < 0.001$) better activity when compared to acarbose and extract obtained from maceration showing significantly ($p < 0.001$) lesser activity when compared to standard drug. This clearly justifies the fact that exhaustive extraction of all possible bioactives present in the plant results in better display of biological potency and in this regard MAE has clearly proved its supremacy by exhibiting better biological activity and also abolishes any fear that microwave irradiation if carried out at optimum conditions can compromise the biological activity of the plant sample due to degradation threats. Similar pattern in favour of EAF obtained from MAE (significantly better activity compared to standard drug acarbose) was also seen with α-glucosidase activity. In conclusion, it can be stated that among all the fractions, the most active was found to be ethyl acetate fraction which showed significantly better activity when compared to EAF obtained from other extraction methods and also when compared to acarbose for both enzyme inhibitory activities.

The results authenticate that a high phenolic, flavonoid, triterpenoid and tannin content are necessarily associated with a strong potency of the tested *D. melanoxylon* leaf fractions against diabetes.

The ethyl acetate fraction from each extraction method exhibited strong i.e., ≥50% inhibition against α-amylase and α-glucosidase activity. Plots of percent inhibition vs log concentration of fractions showed typical sigmoidal dose response curves (Figs. 4 and 5). It was noted that EAF exhibited an IC_{50} value less than acarbose suggesting that it could be a promising lead fraction. It could thus be speculated that this fraction possess significant

Table 2
Results of total polyphenol content (TPC), total flavonoid content (TFC), total triterpenoid content (TTC), and total tannin content (TTC) of different fractions of different extraction method of leaf of *diospyros melanoxylon*.

Treatment		Total polyphenol compounds as mg Gallic acid equivalent (mg GAE/g sample)	Total flavonoid compounds as Quercetin equivalent (mg QE/g sample)	Total Triterpenoid Compounds as Lupeol equivalent (mg LPE/g sample)	Total Tannin Compounds as tannic acid equivalent (mg TAE/g sample)
Soxhlet extract	NHF	11.12 ± 0.34	353.12 ± 6.56	14.36 ± 0.15	12.49 ± 0.17
	CF	9.56 ± 0.33	291.65 ± 7.20	12.54 ± 0.21	10.3 ± 0.18
	EAF	19.52 ± 0.29	847.88 ± 8.30	22.4 ± 0.21	20.56 ± 0.18
	AQF	15.40 ± 0.37	641.56 ± 6.21	16.30 ± 0.16	14.2 ± 0.16
Maceration extract	NHF	8.31 ± 0.14	256.17 ± 4.88	12.26 ± 0.22	10.32 ± 0.33
	CF	7.73 ± 0.18	202.68 ± 7.75	10.72 ± 0.26	8.3 ± 0.21
	EAF	15.49 ± 0.29	786.81 ± 3.74	19.61 ± 0.20	18.38 ± 0.26
	AQF	12.17 ± 0.08	591.63 ± 2.84	16.32 ± 0.37	12.58 ± 0.24
Ultrasound extract	NHF	9.63 ± 1.02	303.82 ± 5.15	13.65 ± 0.24	11.52 ± 0.18
	CF	8.79 ± 0.47	235.86 ± 5.57	11.68 ± 0.32	9.6 ± 0.25
	EAF	17.84 ± 1.13	829.26 ± 6.30	21.34 ± 0.41	19.74 ± 0.31
	AQF	14.54 ± 0.32	625.45 ± 4.07	17.30 ± 0.28	13.9 ± 0.19
Microwave assistant extract	NHF	10.64 ± 0.23	363.64 ± 10.55	13.28 ± 0.12	11.51 ± 0.12
	CF	9.40 ± 0.18	297.96 ± 7.92	13.04 ± 0.12	10.94 ± 0.09
	EAF	21.48 ± 0.19	905.88 ± 14.90	24.59 ± 0.12	23.81 ± 0.06
	AQF	16.37 ± 0.29	660.18 ± 9.47	15.41 ± 0.18	15.59 ± 0.12

The values are expressed as mean of three different experiments ± standard deviation.

Table 3
Results (IC_{50}) of α-amylase and α-glucosidase inhibitory activity of different fractions obtained from different extraction method of *diospyros melanoxylon* leaf.

Treatment		α-amylase inhibitory activity (μg/ml)	α-glucosidase activity (μg/ml)
Soxhlet extract	NHF	164.30 ± 2.57	172.09 ± 1.70
	CF	172.89 ± 1.63	174.93 ± 1.71
	EAF	67.30 ± 0.75[d1,e]	86.24 ± 1.03[**]
	AQF	106.29 ± 0.92	103.28 ± 1.20
Maceration extract	NHF	201.06 ± 2.71	176.48 ± 0.67
	CF	225.93 ± 2.28	181.62 ± 1.35
	EAF	94.52 ± 0.85[a,b2,c2,d2]	116.17 ± 1.12[*, **]
	AQF	145.71 ± 0.54	126.49 ± 0.78
Ultrasound extract	NHF	189.67 ± 2.00	161.38 ± 0.95
	CF	204.08 ± 1.74	159.32 ± 1.34
	EAF	74.27 ± 0.89[d1,e]	93.24 ± 1.10[*, **]
	AQF	120.56 ± 0.95	111.29 ± 1.22
MAE extract	NHF	141.67 ± 1.36	156.66 ± 1.43
	CF	170.12 ± 1.28	185.08 ± 1.56
	EAF	52.39 ± 1.21[a,b1,c1,e]	73.52 ± 1.13[*, **]
	AQF	96.34 ± 0.82	89.21 ± 1.08
Acarbose		72.22 ± 0.94	87.36 ± 0.86

Each value in the table is represented as Mean ± SD (n = 3).
[a]p<0.001 statistically significant as compared to acarbose.
[b1]p,0.01;
[b2] p < 0.001 as statistically significant as compared to EAF fraction of Soxhlet extract.
[c1] p<0.01;
[c2] p < 0.001 statistically significant as compared to EAF fraction of ultrasound extract.
[d1] p<0.01;
[d2] p < 0.01 statistically significant as compared to EAF fraction of MAE extract.
[e] p < 0.01 statistically significant as compared to EAF fraction of maceration extract.
[*]p < 0.001 statistically significant as compared to acarbose.
[**]p < 0.001 statistically significant.

antidiabetic activity and microwave assisted extraction is the best method for the preparation of said fraction (see Fig. 6).

4. Conclusion

In a nutshell it can be stated that MAE is an efficient technique for the preparation of ethyl acetate fraction of the said plant which can be developed as lead fraction for the holistic management of diabetes. Hence the proposed method can be very useful in case of chemical standardization of botanicals as per global standards. MAE could save a lot of time and electrical energy when compared with conversional extraction methods. In MAE the quantity of solvent consumed is least which demonstrates its environment friendly feature. This also suggests that it would save the production cost greatly. In future the proposed extraction method can be called as green extraction method with an ecofriendly edge. In addition, the green aspect of the total procedure becomes a key feature since research concerning new alternatives and new solvents in chemistry are at the moment, for earth and environment protection, a key challenge that we cannot disregard.

This experiment also supports the traditional use of *D. melanoxylon* to treat numerous diseases. Experimental studies of EAF from various extraction method demonstrated significant antidiabetic activity. To the best of our knowledge, it is the first report for comparison of MAE with other conventional extraction technique for their antidiabetic activity on the basis of α-amylase and α-glucosidase inhibitory action obtained from *D. Melanoxylon.* The results of this experiment proved that *D. Melanoxylon* could

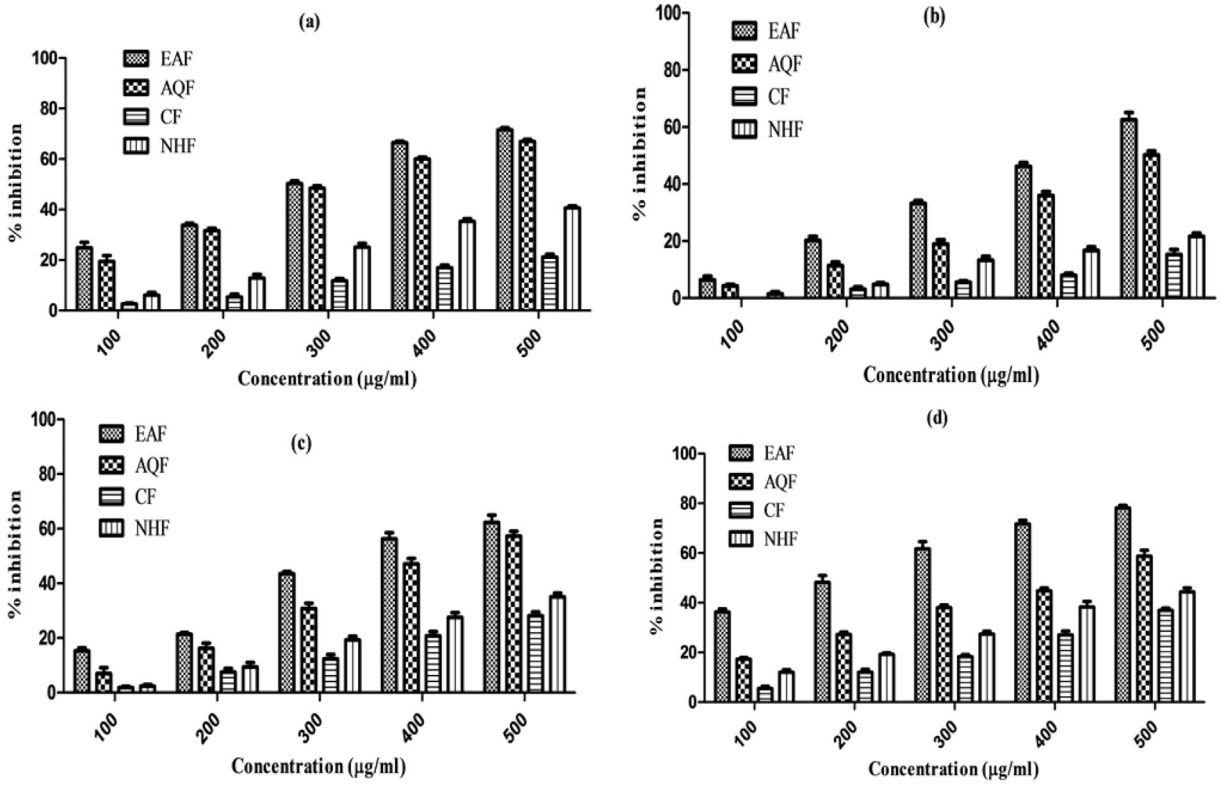

Fig. 4. Alfa amylase activity of different samples on (a) Soxhlet extract, (b) Maceration extract, (c) Ultrasound extract and (d) Microwave assistant extract. Each value is presented as mean ± standard deviation (n = 3).

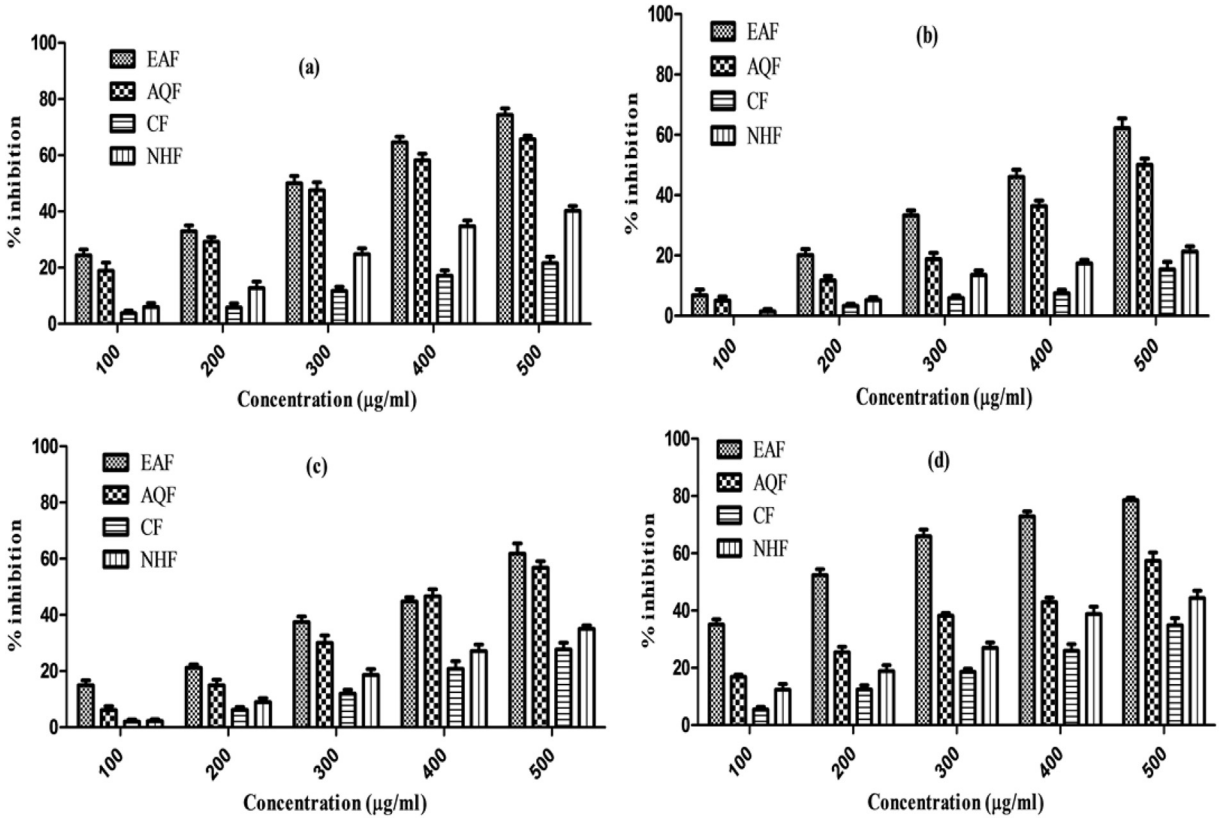

Fig. 5. Alfa glucosidase activity of different samples on (a) Soxhlet extract, (b) Maceration extract, (c) Ultrasound extract and (d) Microwave assistant extract. Each value is presented as mean ± standard deviation (n = 3).

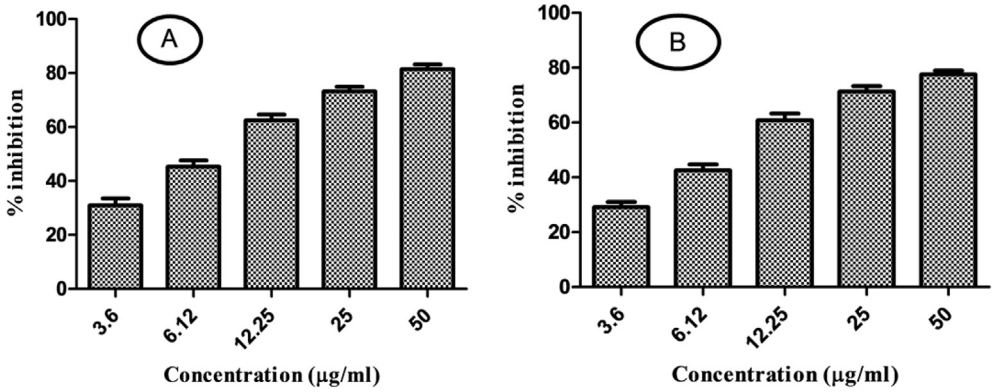

Fig. 6. Standard curve of acarbose for (A) α-amylase and (B) α-glucosidase. Each value is presented as mean ± standard deviation (n = 3).

have a promising appliance in the pharmaceutical and nutraceutical fields due to its rich content of various bioactive compounds. The specific compounds responsible for *D. melanoxylon* biological activities need to be found and additional tests for the most active compounds will be prepared in the near future.

Conflict of interest

The authors have no conflict of interest.

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