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ORIGINAL ARTICLE

Evaluation of Chemical composition and Promising Anti-diabetic activity of essential oil extracts from stems of *Rapanea wightiana* (Wall. ex DC.)

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ABSTRACT
The aim of this study is to evaluate the promising anti-diabetic activity of essential oil and extracts from stems of Rapanea wightiana. The bioactive phytoconstituents from essential oil in various seasons was determined by GC and GC-MS where, a notable change in the composition was observed across all the seasons. The extracts were tested for the enzyme inhibition where, the essential oil, ethanol and ethyl acetate extracts showed significant inhibition with IC_{50} of 79.17, 91.48 and 116.33 µg/ml for α-amylase and 81.29, 98.24.08 and 121.76 µg/ml for α-glucosidase. Subsequently the essential oil and extracts were tested for cytotoxicity using MTT assay HepG2 cells at 0-200µg/ml concentration range. The significant observation was that all the tested samples displayed low level of toxicity at all concentrations in a dose-dependent manner with <40% cell death. Further, in in silico studies, the binding affinities of few major compounds or both compounds were analyzed and found to be excellent in line with the standards used in the respective enzyme inhibition studies. The results reveals that the potential anti-diabetic activity of extracts through inhibition of major enzymes of carbohydrate metabolism and can be explored as viable, reliable and natural alternatives for the synthetic oral hypoglycemic drugs.
Keywords: *Rapanea wightiana, essential oil composition, stem extracts, anti-diabetic activity, α-amylase inhibition, α-glucosidase inhibition and Cytotoxicity*

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INTRODUCTION

Diabetes mellitus (DM) is a disorder of insulin metabolism marked by persistent hyperglycemia and a number of macrovascular and microvascular complications. About 10% of cases are type-1 (T1DM), whereas 90% of cases are type 2 (T2DM). The problems associated with type 2 diabetes have led to a notable reduction in life expectancy [1-2]. Recent research states that 1 in 10 persons have diabetes, of which almost half are undiagnosed, accounting for 537 million people, and diabetes is a factor in 1 in 9 fatalities [3]. It is anticipated that these numbers would keep rising. Most nations are thought to spend between five and twenty percent of their healthcare budgets on diabetes. Diabetes and its consequences cost the world economy US$760 billion in 2019 and are expected to cost US$825 billion by 2030 [3]. An enhanced strategy to the treatment of T2DM is necessary in light of the alarming rise in the prevalence of the disease [4].

The traditional approach to managing diabetes involves changing a patient's lifestyle to reduce or eliminate risk factors like obesity, hypertension, and hyperlipidemia [5]. Healthcare professionals also

employ hypoglycemic medications to help patients manage their diabetes. Different goals are involved in the use of antihyperglycemic medicines to treat hyperglycemia in diabetic patients. For instance, glipizide and other more recent secretagogues, such as chlorpropamide, boost the production of insulin by obstructing the pancreatic β-cell's K+-ATPase channel. Biguanides, like metformin, work by increasing glycogenesis with enhanced insulin sensitivity and inhibiting hepatic gluconeogenesis [6]. Insulin sensitizers selectively bind to peroxisomal proliferator-activated receptor gamma (PPARγ) to increase the effects of insulin on muscle, adipocytes, liver, and other tissues. Additional options include α-glucosidase inhibitors, such as acarbose, which reduce postprandial plasma glucose by competitively inhibiting pancreatic α-amylase and intestinal α-glucosidase. Binding to the pancreatic glucagon-like peptides-1 (GLP-1) receptors, incretin mimetics (like exenatide) regulate the amount of insulin secreted after a meal by increasing the amount of glucose-dependent insulin secreted by the β-cells [7]. In individuals with T2DM, exenatide also increases islet neogenesis and β-cell proliferation in addition to restoring first-phase insulin secretion. Insulin has only been used in dire circumstances to provide rapid glycemic control. These methods have unquestionably improved the care of diabetics throughout time, but there are still a number of drawbacks, including low efficacy, side effects, and expensive therapy [8]. Since ancient times, MAPs (Medicinal and Aromatic Plants), as well as their compounds, have been used to treat a variety of metabolic illnesses. Studies on phytoconstituents from different plants have demonstrated that they scavenge free radicals and reactive oxygen species to control the damage caused by oxidative stress [9-10]. According to earlier research, there may be a relationship between illness incidence and nutrition, with increased intake of foods strong in antioxidants being linked with a lower chance of degenerative disease onset [11-13].

The Myrcinaceae family tree *Rapanea wightiana* (*R. wightiana*), can reach a height of ten meters. It is extensively dispersed throughout South India and Sri Lanka, particularly in the Western Ghats of the Sahyadri and Nilgiri regions. Regarding the plant's therapeutic qualities, it is recommended for a number of respiratory, cardiovascular, and muscular ailments due to its exceptional astringent qualities [14]. The current study is to assess the promising anti-diabetic activity of essential oil and extracts from stems of *R. wightiana* and here in report our results.

MATERIAL AND METHODS

Plant material

The stems of *R. wightiana* were collected in all the four seasons Chikmagalur, Karnataka, India (13° 19' 12.7524'' N 75° 46' 3.27'' E) in 2022.

Solvent extraction

The stems of *R. wightiana* was subjected to extraction using ethanol and ethyl acetate solvents, evaporated the excess solvent using rotary evaporator and stored at 4°C for further experimentation purpose.

Essential oil composition

The essential oil was obtained by hydrodistillation in a Clevenger apparatus and the subjected to GC and GV-MS analysis for determination of chemical composition.

GC Analysis

The analysis was conducted using a Varian-gas chromatograph that was fitted with a FID and a BP-1 capillary column (30m X 0.2mm i.d., film thickness 0.25µm). The carrier gas was helium, and the input pressure was set at 8 p.s.i. at a flow rate of 1.0 ml/min. For 6min., the temperature was designed to rise from 60°C to 220°C at a rate of 5°C per minute. While, injector and detector temperatures were maintained at 250°C and 300°C respectively. The samples (0.2 µl) were injected using a split ratio of 1:100.

GC-MS Analysis

The analysis was conducted using an Agilent 6890 GC equipped with a mass selective detector (5973 N) and a column (HP-5 MS, 30m X 0.25mm X 0.25µ). With a 5 min. hold, the oven temperature was raised from 50°C to 280°C at a rate of 4°C/min (programmed analysis). Inlet and interface temperatures were 250°C and 280°C, respectively. Helium was the carrier gas, flowing at a rate of 1.0 ml/min. The samples (0.2 µl) were injected using a split ratio of 1:120. The quadrupole and ion source temperatures were kept at 150°C and 230°C, respectively.

Identification of compounds

By comparing the retention indices (RI) of the peaks using a BP-1 capillary column, the constituents present in the oil were identified. For this purpose, the standard reference was a saturated mixture of C_8–C_{22} n-alkanes. Additionally, linear interpolation was used for the identification [15-16]. Further molecules identification was facilitated by comparison of mass spectra with Wiley and NIST databases [16-17].

α-amylase inhibitory activity

This enzyme inhibition assay was performed as per the published method [18] with slight. Essential oil and Extracts (0-100 µg/ml; 250µl) were mixed with α-amylase (0.5 mg/ml) in phosphate buffer (0.02 M; pH 6.9; 250 µl) and starch (1%) as a substrate followed by incubation for 10 min. at 37°C. Termination of reaction was by using DNS (dinitro-salicylic acid) reagent (500µl). The absorbance was recorded using UV-VIS spectrophotometer SL-159 at 540nm. The mixture without samples and Acarbose were served as negative and positive control respectively. All the assays were performed in quintuplicate and the α-amylase inhibition was determined by using the formula

$$\% \text{ Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

α-Glucosidase inhibition assay

The α-Glucosidase inhibition assay was carried out according to the published method with slight modifications [19]. Briefly, α-glucosidase (1.0U/ml, 100µl), 50 μl of the extracts (10-100 µg/ml) and 50 μl of 3.0mM pNPG were mixed thoroughly and incubated at 37°C for 30min and terminated with 0.1M sodium carbonate (2 ml). The product, p-nitrophenol released (yellow colour) was measured in UV-VIS spectrophotometer SL-159 at 405 nm. In this assay, acarbose served as a positive control. The α-glucosidase inhibition percentage was determined by the formula

$$\% \text{ Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Cell Lines, Media, chemicals, Reagents and maintenance

All the chemical reagents used were purchased from Sigma Aldrich, India and of analytical grade. The HepG2 liver cells were obtained from iBiolabs, India and subsequently maintained at R.B.V.R.R. Women's College, Hyderabad. The media required for culture were procured from Himedia, India. HepG2 cells were maintained in RPMI 1640 growth medium supplemented with 10% FCS (fetal calf serum) and subsequently incubating 37°C at desired humidity by providing 5% CO_2. The cell line was sub-cultured after the required confluence (90%) was accomplished.

Cytotoxicity Assay

The cytotoxicity of essential oil and extracts were assessed by the method of Mosmann [20]. The HepG2 cells (100 µl, 8,000 cells/well) were seeded into 96-well plates prior to the experimentation and left overnight for adhesion. Then 100 µl of essential oil and extracts at concentration of 10-200 µl / ml / well were added followed by incubation at 37°C for 48h. Then DMEM medium (100 µl containing 10% foetal calf serum) and 0.5 mg/ml MTT was added and then incubated for 3h at the same temperature. The MTT crystals from the medium were dissolved in DMSO (200 μl/well) and recorded the absorbance in a microplate reader (Lab systems) at 540nm. The cytotoxicity was measured as control % (medium only) and the cell death % was determined using the formula

$$\% \text{ Cell death} = 1 - \frac{Absorbance\ of\ testwell}{Averageof\ untreated} \times 100$$

Selection of proteins targets

The Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.pdb.org) provided the three-dimensional X-ray crystallographic structures of two proteins that are known to be crucial in diabetes. The files were saved in the.pdb format. For molecular docking experiments, proteins with the PDB IDs of alpha glucosidase (3L4T) and alpha amylase (3BAX) were utilized as the targeted diabetic receptor proteins. The proteins were retrieved in.pdb format from the www.rcsb.org website.

Preparation of ligand

The three-dimensional structure of the molecules was saved in.pdb format, and canonical SMILES of the ligands major compounds were from PubChem chemical database (https://pubchem.ncbi.nlm.nih.gov/). Acarbose was the medication against which the ligand was compared.

Molecular docking analysis

The CB Dock web server (https://cadd.labshare.cn/cbdock2/php/index.php) was used to perform molecular docking between the ligand and the receptors.15. Generic evolutionary algorithms are used by CB Dock2 (fexible docking approach). When protein was uploaded to the CB Dock web server, water molecules and other heteroatoms were automatically eliminated. The standard docking option was used

to bind each ligand to one of the six target proteins. The encounters were graded based on their docked energy, and docked poses were compared as per the previously published method [21].

RESULTS AND DISCUSSION

Essential oil composition

The essential oil obtained in various seasons from stems of *R. wightiana* was pale yellow in colour with a yield of 0.27%. As evident from Table-1, limonene content varied between 4.18-7.43%, α-pinene 1.02-1.86%, trans-β-ocimene from 2.89 – 3.49%. The variation in other monoterpenes was not very significant. Among oxygenated monoterpene, variation of isofenchol from 1.74 – 2.19%, isopulegol 1.19 0- 2.21%, dihydrocarveol 1.94 – 2.41%, isobornyl acetate 3.98 – 4.55%, methyl cinnamate 1.27 – 1.82% and geranyl acetate 1.75 – 2.11% was observed. However, Methyl eugenol was found to be in higher amounts where it varied between 3.98-4.49% in various seasons. The relative levels of sesquiterpene hydrocarbons were maximum of 26.9% in spring season and a low level of 19% in rainy season. Sesquiterpenes such as β-elemene, germnacrene D, giejerene, β-caryophyllene and pregeijerene were observed as major components, all of which showed substantial seasonal differences. Concerning oxygenated sesquiterpenes, nerolidol varied between 1.08 – 1.93%, β-bisabolol 1.57 – 1.79%, caryophyllene oxide 0.97 – 1.97% and farnesol 3.11 – 3.85%. The significant observation was that τ-cadinol was absent in winter and spring seasons in stem oil while spathulenol was not detected in summer season.

Enzyme inhibition and cytotoxicity

The results depicted in Table-2 offer the evaluation and presentation of the extracts' inhibition of the α-amylase enzyme. The oil and extracts exhibited excellent inhibition with IC_{50} values of 79.17, 91.48 and 116.33 µg/ml respectively for essential oil, ethanol and ethyl acetate extracts suggesting that all the samples a remarkable inhibitory efficacy. Acarbose displayed an IC_{50} of 29.26 µg/ml. The assay was conducted at several concentrations ranging from 0-100 µg/ml. With increase in the concentration, there was a discernible rise in the extracts' inhibitory percentage. Similarly, the oil and extracts displayed excellent inhibition of α-glucosidase enzyme. The IC_{50} values recorded were 81.29, 98.24.08 and 121.76 µg/ml respectively (Table-3). While, Acarbose, the positive control, showed an IC_{50} of 31.36 µg/ml. The assay employed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for the cytotoxic studies *in vitro* of extracts on HepG2 cells at varied concentrations from 0-200µg/ml. Both the extracts displayed low level of toxicity at all concentrations in a dose-dependent approach (Fig-1-3). However, the extracts exhibited <40% cell death even at the maximum concentration of 200 µg/ml.

It is known that the compounds functioning as α-amylase and α-glucosidase enzyme inhibitors slow down the small intestine's breakdown of carbohydrates (catabolism) and lower blood glucose levels after meals and forms an important approach for maintenance of glucose levels [22-23]. Potential α-amylase and α-glucosidase inhibitory actions have been reported for a wide range of botanicals and phytoconstituents [22]. The study's findings regarding extracts and oil under investigation demonstrated their strong inhibition of the enzymes *α*-amylase and *α*-glucosidase, which is consistent with reports that have been published. Furthermore, it has been reported and demonstrated that phytoconstituents such terpenoids are potent inhibitors of the enzymes α-amylase and *α*-glucosidase [24-27]. The extracts and oil under investigation exhibited potent anti-diabetes activity by inhibition of the two enzymes which are in accordance with those of the published reports. The cytotoxicity of essential oils, and extracts were assessed on HepG2 cells and the results specify that all the samples exhibited less than 40% cell death even at highest concentration of 200 µg/ml. All the samples displayed very low level of toxicity in comparison with the plants described and published in the literature which have been presented > 50% cell death [28-29]. This aspect is very encouraging in the use of this botanical in clinical and herbal formulations.

Table-1: Chemical composition of essential oil from stems of *R. wightiana* in various seasons

S. No.	Compound	RI	% Composition in various seasons				MOI
			Summer	Rainy	Winter	Spring	
1.	α-pinene	937	1.86±0.37	1.74±0.29	1.28±0.22	1.02±0.26	a, b, c
2.	Sabinene	977	1.08±0.22	1.27±0.33	2.28±0.35	2.04±0.38	a, b, c
3.	β-pinene	981	0.98±0.02	0.79±0.03	2.71±0.29	2.57±0.31	a, b, c
4.	Myrcene	984	0.94±0.03	1.24±0.06	1.38±0.09	1.63±0.09	a, b, c
5.	α-phellendrene	1009	1.32±0.12	1.45±0.11	1.63±0.18	1.72±0.17	a, b, c
6.	α-terpinene	1020	1.98±0.14	2.79±0.34	2.44±0.37	2.33±0.31	a, b, c
7.	Limonene	1024	6.47±0.41	7.43±0.39	4.18±0.38	4.26±0.36	a, b, c
8.	Cis-β-ocimene	1035	0.91±0.02	1.18±0.11	1.19±0.16	1.12±0.14	a, b, c
9.	Trans-β-ocimene	1040	3.38±0.33	3.49±0.41	3.21±0.32	2.89±0.27	a, b, c

10.	Linalool	1085	0.87±0.03	0.89±0.03	0.69±0.02	0.64±0.06	a, b, c
11.	Isofenchol	1101	1.74±0.21	2.19±0.22	2.06±0.26	2.01±0.24	a, b, c
12.	Sabinene hydrate	1116	0.82±0.03	0.99±0.05	0.90±0.07	0.81±0.06	a, b, c
13.	Geijerene	1143	-	-	3.11±0.42	4.98±0.44	a, b, c
14.	Isopulegol	1146	1.22±0.31	1.19±0.22	2.07±0.28	2.21±0.29	a, b, c
15.	Isothujanol	1157	1.67±0.29	1.78±0.29	2.11±0.31	2.15±0.33	a, b, c
16.	Terpinene-4-ol	1166	0.88±0.07	0.76±0.08	0.54±0.05	0.38±0.09	a, b, c
17.	Borneol	1180	1.24±0.35	1.36±0.21	1.57±0.26	1.47±0.19	a, b, c
17.	Decanal	1192	2.61±0.33	2.17±0.36	2.10±0.25	1.99±0.29	a, b, c
18.	Dihydrocarveol	1195	1.94±0.18	2.33±0.22	2.16±0.27	2.41±0.28	a, b, c
18.	Geraniol	1240	0.99±0.09	1.04±0.08	1.22±0.11	0.97±0.11	a, b, c
19.	Isobornyl acetate	1271	3.98±0.34	4.23±0.33	4.08±0.36	4.55±0.31	a, b, c
20.	Pregeijerene	1285	-	-	3.26±0.29	4.62±0.27	a, b, c
21.	Delta elemene	1337	0.78±0.06	0.93±0.07	0.91±0.07	0.90±0.09	a, b, c
22.	Methyl cinnamte	1342	1.69±0.13	1.72±0.18	1.82±0.12	1.27±0.18	a, b, c
23.	Geranyl acetate	1370	1.75±0.22	2.11±0.19	2.08±0.29	1.89±0.26	a, b, c
24.	β-Bourbonene	1386	0.56±0.02	1.09±0.04	1.14±0.07	1.34±0.09	a, b, c
25.	β-Elemene	1389	1.82±0.11	1.82±0.13	1.77±0.17	1.46±0.15	a, b, c
26.	Methyl eugenol	1403	4.49±0.32	4.21±0.33	4.21±0.29	3.98±0.31	a, b, c
27.	β-caryophyllene	1421	1.54±0.17	1.72±0.16	1.49±0.17	1.31±0.19	a, b, c
28.	α-humulene	1446	1.19±0.21	1.28±0.19	1.23±0.11	1.30±0.13	a, b, c
29.	Germacrene D	1480	8.97±0.37	3.62±0.23	4.55±0.26	6.67±0.39	a, b, c
30.	D-cadinene	1536	2.02±0.13	2.32±0.22	2.27±0.23	2.14±0.27	a, b, c
31.	Nerolidol	1544	1.93±0.12	1.88±0.15	1.28±0.16	1.08±0.14	a, b, c
32.	Spathulenol	1564	-	2.76±0.23	2.62±0.24	2.34±0.21	a, b, c
33.	Caryophyllene oxide	1574	1.97±0.14	1.44±0.21	1.26±0.17	0.97±0.06	a, b, c
34.	τ-cadinol	1642	1.38±0.11	0.98±0.08	-	-	a, b, c
35.	β-bisabolol	1672	1.57±0.14	1.75±0.17	1.66±0.13	1.79±0.21	a, b, c
36.	Farnesol	1699	3.85±0.26	3.79±0.28	3.83±0.29	3.11±0.23	a, b, c
37.	Farnesyl acetate	1790	-	0.59±0.04	-	-	a, b, c

MOI – Method of Identification

a – Retention time; b – Retention indices; c – Mass spectra

Table-2: α-amylase inhibition potential of oil and extracts of *R. wightiana*

Compound/Extract	IC_{50} value (µg/ml)	IC_{90} value (µg/ml)	Relative efficacy
Essential oil	79.17± 2.13**	146.43± 3.87**	0.369
Ethanol Extract	91.48 ± 2.97*	171.19± 4.03*	0.319
Ethyl acetate extract	116.33 ± 3.49*	213.48± 4.87*	0.251
Acarbose	29.26 ± 0.92**	51.34± 1.27**	1

Values expressed as mean ±SE; *p<0.05, **p<0.01

Relative efficacy = IC_{50} of standard/IC_{50} of sample

Table-3: α-glucosidase inhibition potential of oil and extracts of *R. wightiana*

Compound/Extract	IC_{50} value (µg/ml)	IC_{90} value (µg/ml)	Relative efficacy
Essential oil	81.29± 1.86**	150.08± 3.42**	0.385
Ethanol Extract	98.24 ± 2.03*	185.63± 3.88*	0.319
Ethyl acetate extract	121.76 ± 2.71*	225.19± 4.11*	0.257
Acarbose	31.36 ± 1.03**	54.72± 1.21**	1

Values expressed as mean ±SE; *p<0.05, **p<0.01

Relative efficacy = IC_{50} of standard/IC_{50} of sample

Table-4: Docking studies of major bioactive compounds with different enzymes

S.No	Name of the compound	Name of the enzyme	PDB ID	Vina score
1.	Limonene	Alpha amylase	3BAX	-7.4
		Alpha glucosidase	3L4T	-7.2
2.	Trans-β-ocimene	Alpha amylase	3BAX	-7.6
		Alpha glucosidase	3L4T	-7.8
3.	Isobornyl acetate	Alpha amylase	3BAX	-6.3
		Alpha glucosidase	3L4T	-6.5
4.	Methyl eugenol	Alpha amylase	3BAX	-6.6
		Alpha glucosidase	3L4T	-6.9
5.	Germacrene D	Alpha amylase	3BAX	-5.4
		Alpha glucosidase	3L4T	-5.9
6.	Farnesol	Alpha amylase	3BAX	-6.0
		Alpha glucosidase	3L4T	-6.2
7.	Acarbose	Alpha amylase	3BAX	-7.6
8.	Acarbose	Alpha glucosidase	3L4T	-6.9

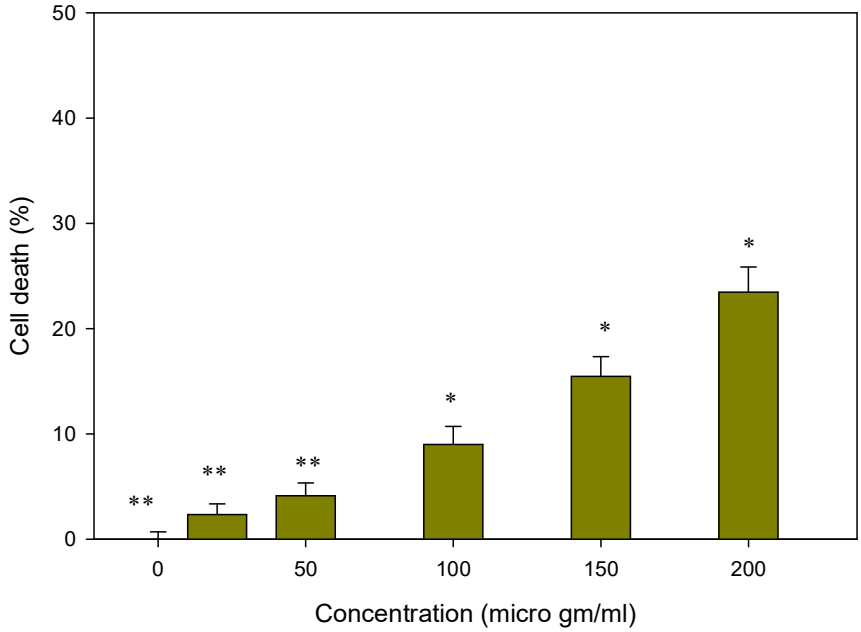

Fig-1 Cytotoxicity assay of the stem essential oil on HepG2 liver cells

Data expressed as % control ± SE; *p<0.05, **p<0.01

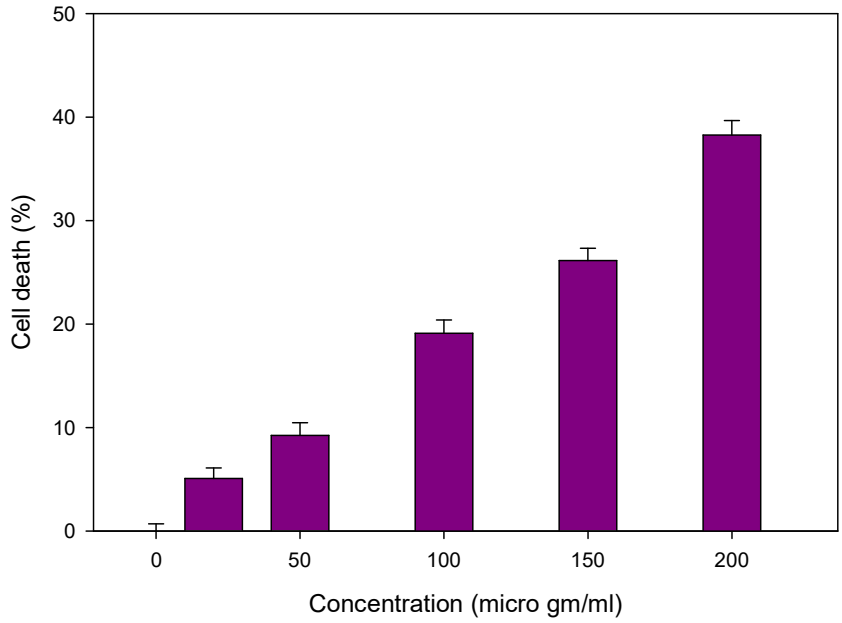

Fig-2: Cytotoxicity assay of the Ethanol extract on HepG2 liver cells

Data expressed as % control ± SE; *p<0.05, **p<0.01

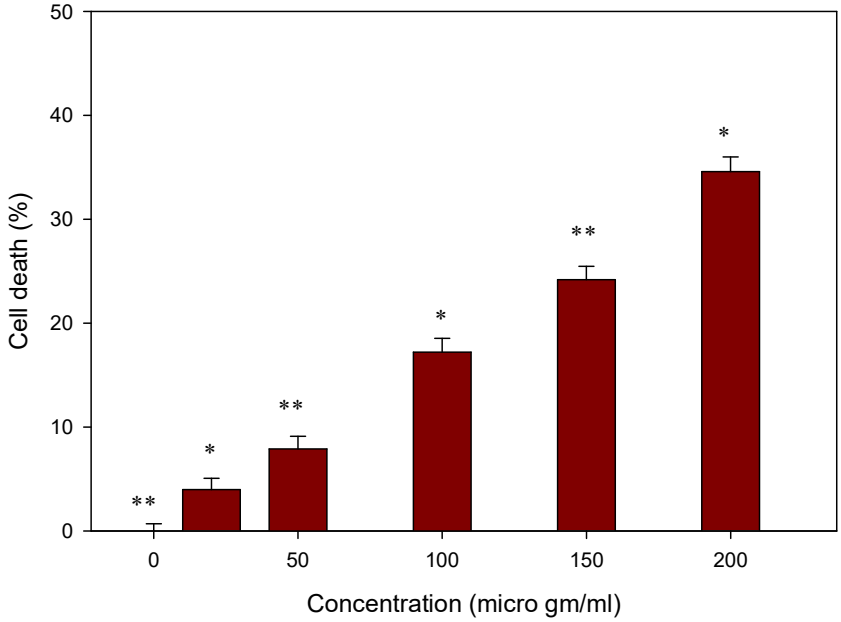

Fig-3: Cytotoxicity assay of the Ethyl acetate extract on HepG2 liver cells

Data expressed as % control ± SE; *p<0.05, **p<0.01

***In silico* molecular docking**

The major compounds have been subjected to molecular docking where, this simulation allowed the investigation of the behaviour of compounds on α-amylase and α-glucosidase inhibition. A good binding affinity for all the compounds were observed compared favourably with acarbose in the respective enzyme inhibition studies (Table-4). These results reveals that the extract and compounds possess significant antidiabetic potential and can be extended for a suitable formulation for the management of diabetes. Research on novel compounds with pharmacologic activity may benefit from the use of terpenoids, a promising source of naturally occurring molecules with biological activity. This compound's

action on the carbohydrate-binding regions of α-amylase and α-glucosidase enzymes, which catalyze the α-1,4 glycosidic linkages hydrolysis may form a mechanistic approach by which these phytoconstituents exert their efficacy and act as natural hypoglycemic agents.

CONCLUSION

The essential oil and extracts exhibited excellent anti-diabetic activity via inhibition of α-amylase and α-glucosidase, the two important carbohydrate metabolizing enzymes. and this can be ascribed to the presence of substantial number of phenols, flavonoids, alkaloids and sesquiterpenes. Further, in cytotoxicity studies on HepG2 cells using MTT assay, the oil and extracts displayed low level of toxicity at all the tested concentrations in a dose-dependent manner with <40% cell death. Further research is underway to separate the phytoconstituents and evaluate their efficacy using in vivo mice models where, the information obtained might help in the development of nature lead molecules and act as little helpers in the management of diabetes.

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COMPETING INTERESTS

The authors have declared that no competing interest exists

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