PHYTOCHEMICAL STUDY AND IN VITRO ANTI-DIABETIC ACTIVITY OF EUCALYPTUS AND LEMON OILS

Yogendra Singh[1], Karan Kumar Das[2], Dr. Mathews T Thelly[3], Dr. Bhupendra Kumar Kumawat[4], Dr. R. Soruba[5], Roshan Kumar[6], M. Narayani[7], Dr. Rama Prasad Padhy[8], Ayushmaan Roy[9]**

[1]Director, IQAC & Professor, School of Pharmaceutical Sciences, MVN University, Palwal, Haryana, India.

[2]Assistant Professor, Bharat Technology, Uluberia, Howrah, West Bengal, India.

[3]Associate Professor, Head and Research Guide, Department of Botany, Kuriakose Elias College Mannanam 686561, India.

[4]Peofessor & Principal, Northern Institute of Pharmacy & Research, Alwar, Rajasthan, India.

[5]Associate Professor and Head, Department of Plant biology and plant biotechnology, Quaid-e-millath government college for women (autonomous), Chennai- 600002, India.

[6**]Assistant Professor, Guru Nanak College of Pharmaceutical Sciences, Dehradun, India.

[7]Assistant professor, Department of Microbiology, Thassim Beevi abdul kader college for women, kilakarai, Ramanathapuram district, Tamilnadu-623517, India.

[8]Professor cum Vice Principal, Danteswari College of Pharmacy, Borapadar, Raipur Road, Jagdalpur, Chhattisgarh-494221, India.

[9]Assistant professor, Rungta college of Pharmaceutical Sciences & Research, Bhilai, India.

Corresponding Author: Roshan Kumar: Email: rjroshan244@gmail.com

Graphical abstract

Abstract

Research is being done to determine whether or not traditional medicinal plants offer a viable alternative to the use of synthetic medicines in the treatment of diabetes. It is general knowledge that lemon oil and eucalyptus oil can be used as a treatment for a variety of diseases, including diabetes. This study was carried out with the intention of determining the in vitro effects of an ethanol extract of Eucalyptus citriodora (EOE) and lemon oil (LOE), as well as the possible mechanisms of action behind such effects. It was determined whether or whether BRIN BD11 clonal pancreatic -cells and mouse islets were capable of insulin secretion. The effects of EOE and LOE on glucose reduction were further investigated using a variety of additional in vitro models. Because of problems with bioavailability, organoleptic unacceptability, and other factors, the traditional ways of administering herbal medicine frequently result in noncompliance on the part of patients and a reduction in the effectiveness of the drug. This is true despite the immense potential of herbal therapy. It is important, while formulating dosage forms using eucalyptus bio-actives, to keep in mind the hypoglycemic potential of both eucalyptus and lemon. It is possible that the use of eucalyptus and lemon for the treatment of type 2 diabetes mellitus may gain greater momentum if nano-technological methods were utilized for their administration. This would lower the risks of adverse reactions, enhance their bioavailability, and improve their flavor. Additionally, it would improve their bioavailability.

Keywords: Diabetes, Eucalptus, Lemon, Glucose

Introduction

Diabetes mellitus (DM), which refers to a collection of chronic metabolic illnesses, is becoming an increasingly important issue on a global scale. It is currently the seventh leading cause of mortality globally[1]. Nearly 10.5% of the world's population is affected by diabetes mellitus

(DM), the vast majority of whom have type 2 diabetes mellitus (T2DM). Insulin resistance, obesity, chronic inflammation, oxidative stress, and mitochondrial dysfunction are all important factors in the development of type 2 diabetes, which is caused by the pathophysiology of the disease[2]. In addition to these genetic predispositions, lifestyle decisions are also a major contributing to the rising incidence of diabetes, which has now reached epidemic proportions. The body mass index, also known as BMI, is frequently utilized as a quantitative measure of the detrimental consequences that obesity has on human health. In obese people with elevated amounts of nonesterified fatty acids (NEFAs) produced from adipose tissue, there is a potential for insulin resistance and cell dysfunction, both of which can contribute to type 2 diabetes. This raises concerns about the potential for the development of type 2 diabetes[3]. Patients who have type 2 diabetes and are overweight may also have a larger chance of having cardiovascular disease (CVD), which includes coronary artery disease, angina, atrial fibrillation, myocardial infarction, and sudden cardiac arrest. Patients with type 2 diabetes who are not overweight may not have an increased risk of developing CVD[4]. However, at this time, there is no treatment for diabetes; all that can be done is manage the symptoms. There is a species of Eucalyptus that belongs to the family Myrtaceae called lemon eucalyptus. It is also commonly known as lemon-scented gum, blue spotted gum, or eucalyptus citriodora. Because of its analgesic, anti-inflammatory, and antipyretic effects, as well as its efficiency against respiratory infections and sinus congestion, eucalyptus citriodora is used by a significant number of people[5]. It contains a variety of essential oils, including cineole, citronellal, and citronellic acid, to name just a few of the more well-known ones. Inflammatory cells are responsible for the production of cytokines and chemokines such as thromboxane B2, leukotriene B4, and tumor necrosis factor alpha (TNF-a). Essential oils have been shown to inhibit the growth of a variety of microorganisms, including yeasts, fungi, and bacteria, making them effective antibacterial agents. After a meal, the skeletal muscles are only able to utilise roughly two-thirds of the glucose that is present in the blood without the assistance of insulin[6][7] When insulin binds to its receptor, it initiates a cascade of events that ultimately results in GLUT4 being translocated from the cytosol to the plasma membrane. This improves the cell's ability to take in glucose. Tyrosine residues in the INSR subunit are phosphorylated in response to insulin binding, which in turn recruits several substrates, including IRS-1, IRS-2, and phosphoinositide 3-kinase (PI3K). Although skeletal muscle does utilise some glucose, the vast majority of glucose is taken up by hepatocytes and transformed into glycogen by the hormone insulin. Skeletal muscle does consume some glucose. Upon making contact with its receptor, insulin sets off a chain reaction of phosphorylation that affects a number of different metabolic pathways. These metabolic pathways include gluconeogenesis, glycogen synthesis, glycogenolysis, and lipid synthesis. Insulin and glucagon work together to fine-tune these metabolic processes. Insulin is responsible for encouraging glucose storage and glycogen synthesis, whilst glucagon is responsible for promoting glucose generation and glycogen breakdown in the liver[8][9] Together, these two hormones cooperate to fine-tune these metabolic processes. When insulin sensitivity in the liver (also known as hepatic IR) is poor, there is a concomitant decrease in glycogen creation and an increase in hepatic gluconeogenesis, lipogenesis, and the synthesis of proinflammatory proteins like C-reactive protein (CRP). These changes occur at the same time[10]. This can result in chronic inflammation of the liver, which in turn can make IR symptoms worse[11].

After a meal, when insulin binds to its receptor in adipose tissue, the glucose transporter GLUT4 has an easier time doing its job and absorbing glucose. This triggers the beginning of the metabolic pathway that will ultimately lead to the production of glycerol-3-phosphate (G3P). During periods of fasting, the body relies on triacylglycerols, which are formed when G3P is esterified with other fatty acids[12][13]. These are used as a source of energy. Insulin resistance (IR) is characterised by a decrease in glucose absorption, an increase in lipolysis, and a decrease in the efficiency with which fatty acids are taken in from the blood. Adipose tissue can be damaged by IR. IR can also lead to increased risk of type 2 diabetes. When adipose IR causes an abnormal version of AKT to be activated, hyperglycemia is made worse as a result. This abnormal form of AKT stops GLUT4 from being delivered to the membrane and activates lipolytic enzymes. On the other hand, type 2 diabetes is made worse by the buildup of free fatty acids in organs such as the liver[14]. This accumulation can lower insulin sensitivity and increase hepatic gluconeogenesis, both of which contribute to the progression of type 2 diabetes.

Adipose tissue, which has a dynamic endocrine role, is responsible for the secretion of a number of hormone-like compounds that are collectively referred to as adipokines. Increases in the size and/or mass of adipose tissue have been associated to several different pathological processes, including fibrosis, hypoxia, macrophage-mediated inflammation, and pathologic vascularization. A diet high in fat can cause inflammation and malfunction in adipose tissue by activating proteins and transcription factors in the mitochondria[15]. This can happen when someone consumes a diet high in fat. When the size of adipocytes is altered and immune cells penetrate fat tissue, the release of proinflammatory cytokines occurs. Two examples of these cytokines are tumor necrosis factor alpha (TNF-) and interleukins (IL-6 and IL-1), both of which belong to the family of interleukins. This results in metabolic inflammation, which is a chronic inflammatory disease that plays a significant role in insulin resistance as well as type 2 diabetes[16].

After a meal, in addition to the activities that have already been described, the intestine additionally secretes two incretins called glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). These incretins cause an increase in the amount of insulin that is produced by the pancreas[17]. Because of the activity of an enzyme known as dipeptidyl peptidase-4 (DPP-4), the half-life of these peptides is relatively brief. Only GLP-1 has been shown to suppress glucagon release and exhibit growth-factor-like actions on pancreatic -cells, which leads to an increase in insulin gene expression and insulin manufacture. The effects of GLP-1 and GIP on insulin secretion are comparable. Because of this, GLP-1 has recently gained attention as a potentially useful pharmacological target for the research and development of diabetes treatments that are based on its action mimicking[18]. In type 2 diabetes, both the activity and the quantity of incretins are altered, which leads to a decrease in the fed state's glucose-dependent production of insulin. This decrease in insulin production is the result of type 2 diabetes. The pancreas will no longer respond sensitively to GIP, but it will continue to respond sensitively to GLP-1. A rise in the expression of DPP-4 or a fall in the expression of GIP and GLP-1 receptors are also possible explanations for this phenomenon[19].

Material & Methods

Collection & Preparation of Plant Extract

We went to a market close by in search of essential oils containing eucalyptus and lemon. After combining 200 grams of plant oil and one liter of ethanol with a volume-to-volume ratio of 80%, the mixture was shaken at 900 grams for between 48 and 72 hours[20]. The extract was then dehydrated at a temperature of 40 degrees Celsius while under a reduced amount of pressure after first being filtered through Whatman no. 1 filter paper. A semi-solid, gelatinous crude extract of E. citriodora and Lemon oil (6 g) was obtained by vacuum-drying an oily filtrate using a rotary evaporator machine[21]. This extract was then stored at a temperature of 4 degrees Celsius for use in subsequent research. Due to its insolubility in water, EEEC had to be extracted using 60 µL/10 mL of dimethyl sulfoxide with a concentration of 0.6% DMSO. This extraction method was found to be safe to the cells, which allowed the research to continue

Phytochemical Screening of plant Extract [21][22][23]

Tannins, saponins, flavonoids, alkaloids, phenols, glycosides, steroids, and terpenoids were all identified through qualitative confirmatory phytochemical screening of plant extracts using standard techniques.

Test for Tannins

To determine whether or not the plant extract included tannins, approximately 200 mg of the extract was brought to a boil in 10 mL of distilled water, and 0.1% ferric chloride was then added to the mixture.

Test for Alkaloids

Before the plant extract was filtered and heated in steam with two milliliters of the filtrate and three drops of 1% HCl, it was dissolved in one hundred milliliters of water. After that, the filtrate was boiled with the plant extract. After that, one milliliter of the previously heated liquid was combined with six milliliters of the Mayer-Wagner reagent. It was determined that alkaloids were present due to the production of a precipitate that was either white or brownish-red in color.

Test for Saponins

The mixture was agitated while the extract was diluted with five milliliters of water that had been distilled. The formation of foam afterwards served as conclusive evidence for the presence of saponins.

Test for Flavonoids and Glycosides

After mixing 10 mL of ethanol and 200 mg of plant extract, the mixture was strained through a filter. A ribbon of magnesium, two milliliters of the filtrate, and strong hydrochloric acid were mixed together. The appearance of a pink or crimson tint is an indication that flavonoids are present in the sample. After adding 1 mL of distilled water and NaOH to 0.5 mL of the crude

extract, which caused the crude extract to turn a reddish color, the presence of glycosides was demonstrated to be true.

Test for Steroids

After mixing approximately 1 milliliter of the crude extract with 10 milliliters of chloroform and 10 milliliters of sulfuric acid, a bilayer was produced. The top layer of the bilayer was red, and the bottom layer was greenish. This confirmed the existence of steroids.

Test for Terpenoids

The existence of terpenoids was determined by mixing 0.5 milliliters of crude extract, 2 milliliters of chloroform, and 3 milliliters of sulfuric acid, which resulted in the formation of a reddish-brown color.

Test for Phenols

After adding three drops of $FeCl_3$ and one milliliter of K_2Fe (CN_6) to one milliliter of the extract, the mixture was stirred. The manifestation of greenish blue forms served as conclusive evidence for the presence of phenols.

In Vitro Insulin-Releasing Studies [23]

The -glucosidase inhibitory activities were evaluated utilizing methods that had been published in the past, with a few tweaks here and there. During the course of the study on inhibitory activity, the -glucosidase inhibitor acarbose served in the capacity of a standard. We were able to obtain ready-to-use quantities of acarbose, ESH, Q3G, I3G, quercetin, and isorhamnetin after dissolving them in water containing 1% DMSO (the final concentration of DMSO in the reactional mixture was 0.2%). After an incubation period of 15 minutes at 37 degrees Celsius, 80 liters of sample and 100 µl of -glucosidase at a concentration of 0.5 units per milliliter in a phosphate buffer with a pH of 6.8 were mixed together. Following an incubation period of 15 minutes at 37 degrees Celsius, 100 µl of a p-NPG substrate concentration of 5 millimoles per liter was added to the mixture. The process was stopped by adding 100 µL of a sodium carbonate solution at a concentration of 0.2 µM. At a wavelength of 405 nm, the absorbance of p-nitrophenol (pNP) that had been freed from p-nitrophenyl-D-glucopyranoside (pNPG) was measured using a microplate reader made by PowerWave called the PowerWave XS. The following formula was utilized in the determination of the sample's glucosidase inhibition:

α-glucosidase inhibitory activity (%) = $[(A0 - A1)/A0] \times 100$,
where A0 is the absorbance without the sample (control) A1 is the absorbance with the sample.

Glucose uptake [24]

A total of 96-well plates were outfitted with three experimental groups: a blank group, a positive group, and a drug group. Within each of these groups, ten replicates of well-grown L6 cells were planted. Before beginning the experiment, L6 cells were cultured in media devoid of serum for a period of two hours. Subsequently, the cells were subjected to stimulation in an incubator at 37 degrees Celsius and 5% CO2 with 100 nM insulin and various doses of EDP for a duration of half an hour. After being exposed to EDP for thirty minutes, L6 cells initiated a reaction with

glucose oxidase that resulted in the production of red quinone mimide pigment. The amount of red quinone imide pigment generated was proportional to the amount of glucose present in the sample, which made it possible to determine absorbance at 505 nm. After the supernatant and glucose standard had been discarded, a fresh 96-well plate was introduced into the incubator after it had been cleaned. The absorbance value was calculated by setting a microplate reader to a wavelength of 505 nm and then adding 200 µL of glucose oxidase reagent to each well. This resulted in the calculation of the absorbance value. After the effect of the medication had worn off, the medium was thrown away, and 0.5 mg/mL of MTT solvent was added to each well. After that, the wells were incubated in the dark for four hours to determine whether or not the drug was cytotoxic. In order to determine the absorbance value at 492 nm, a microplate analyzer was used after the liquid had been discarded[25]. DMSO solvent was applied to each well of the device. In order to determine how the L6 cells would react to the drug, they were given a high dosage of insulin (1 µM) for one day prior to receiving the medication. In contrast, the control cells were not given any treatment. The glucose assay kits that were utilized in this research were supplied by Tec. Product Inc. of Xiamen, China. The measurement of glucose uptake was carried out in accordance with the procedure for the GLU-OX test (the GOD-POD technique). The analysis of this data was carried out with the assistance of Graphpad Prism 7.

Statistical Analysis

Graph Pad Prism 5 was used to conduct an analysis of the data, and the level of significance for the mean standard error of the mean was judged to be $P < 0.05$. Either the unpaired Student's t-test (non-parametric, two-tailed P values) or the one-way analysis of variance followed by Bonferroni post hoc testing was used to assess all of the data. Both methods yielded the same conclusions. After making corrections for the baseline, we were able to determine the area under the curve by using the trapezoidal principles and calculating the result.

Result & Discussion

Phytochemical Screening

All of the submitted plant extracts showed promising phytochemical findings, as shown by noticeable colour shifts (Table 1). More than half of the plants screened contained chemicals in the flavonoid, alkaloid, or phenol groups. *Eucalyptus* and *lemon* both showed strong evidence of flavonoids' beneficial effects in the form of a noticeable shift in colour.

Table 1. Phytochemical screening of *Eucalptus* oil and *lemon* oil

Phytochemical Screening	*Eucalptus oil*	*Lemon* oil
Carbohydrates	+	+
Protein	-	+
Glycosides	+	+
Flavonoids	+	+
Tannins	+	+
Triterpenoids	+	+
Terpenoids	+	+

Steroids	+	+

In Vitro Insulin-Releasing Studies

BRIN-BD11 cells produced insulin at a baseline rate of 1.20± 0.05 ng/10^6 cells/20 minutes when they were exposed to 5.6 mM glucose. When the positive control alanine (10 mM) was present, the rate increased by 5.97± 0.19 ng/10^6 cells/20 minutes, which was statistically significant (p<0.001; n = 8). Increasing doses of EEEC have been shown to have an influence on the amount of insulin secreted by BRIN-BD11 cells. EEEC produced insulin at a rate ranging from 1.63±0.10 to 5.69± 0.22 ng/10^6 cells/20 minutes in response to glucose concentrations that ranged from 5.6 mM to 5000 g/mL (p<0.05 to 0.001). The insulin secretion rate from the BRIN-BD11 clonal pancreatic cell line at a glucose concentration of 16.7 mM was 1.84± 0.03 ng/106 cells/20 minutes under basal conditions, but it jumped to 9.70± 0.40 ng/10^6 cells/20 minutes when the cells were depolarized (p <0.001 for both conditions). Insulin release was greatly increased by EEEC at 16.7 mM glucose (p< 0.001), going from 2.03± 0.22 ng/10^6 cells/20 minutes at 1.6-5000 µg/mL to 6.53 ±0.29 ng/10^6 cells/20 minutes. This increase was statistically significant. However, there was no detectable effect on the release of lactate dehydrogenase when EEEC concentrations ranged from 1.6 to 200 g/mL. After being treated with EEEC, isolated mouse islets released a significant amount more insulin in response to 16.7 mM glucose. The EEEC demonstrated a statistically significant rise in insulin secretion at a concentration of 25 µg/mL (p <0.001). EEEC insulin secretion was statistically significant (p <0.001) at a concentration of 200 ng/mL, and it was equivalent to that generated by GLP-1 (10^{-6} and 10^{-8} M) at this concentration.

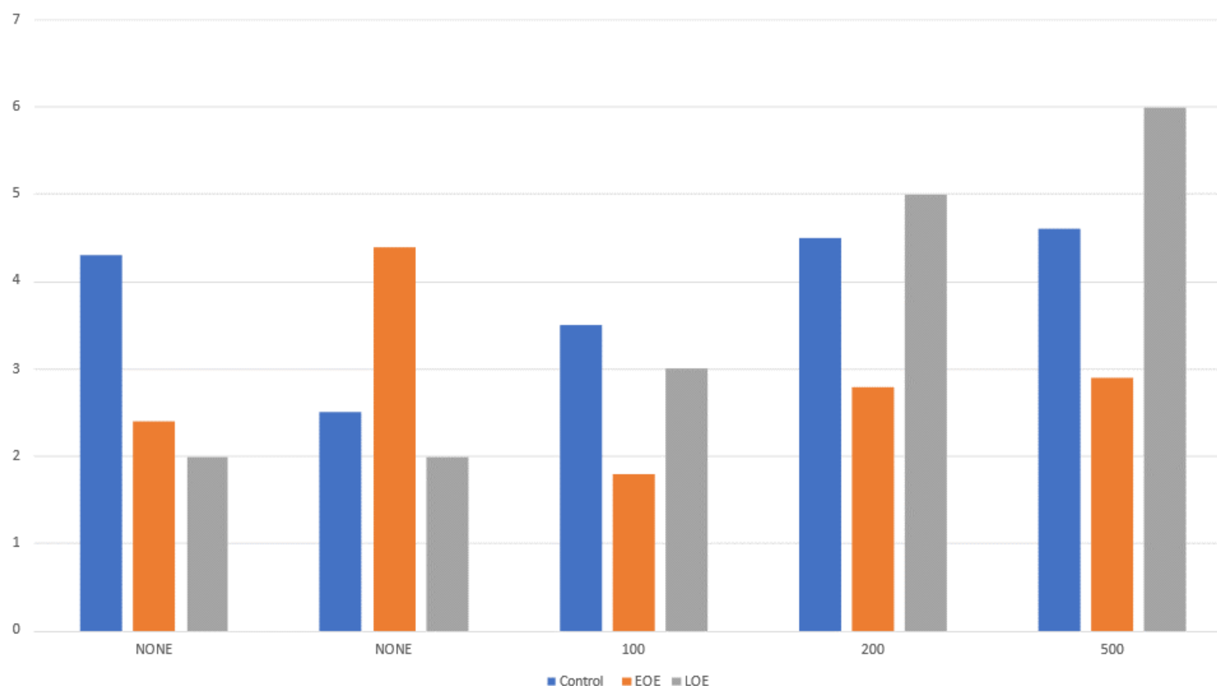

Figure 2. Invitro Insulin release Capacity

Glucose Uptake

Using 3T3L1 differentiated adipocyte cells and a fluorescent glucose analogue called 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose, we studied the effects of EOE and LOE on glucose absorption. Following treatment with EEEC, an increase in glucose uptake was seen ($p<0.05$). Insulin at a concentration of 100 nM increased the efficacy of stimulation ($p<0.001$) When insulin was present, the body absorbed approximately 45% of the glucose that was consumed, but when EEEC was utilized in its place, that number plummeted to 34%.

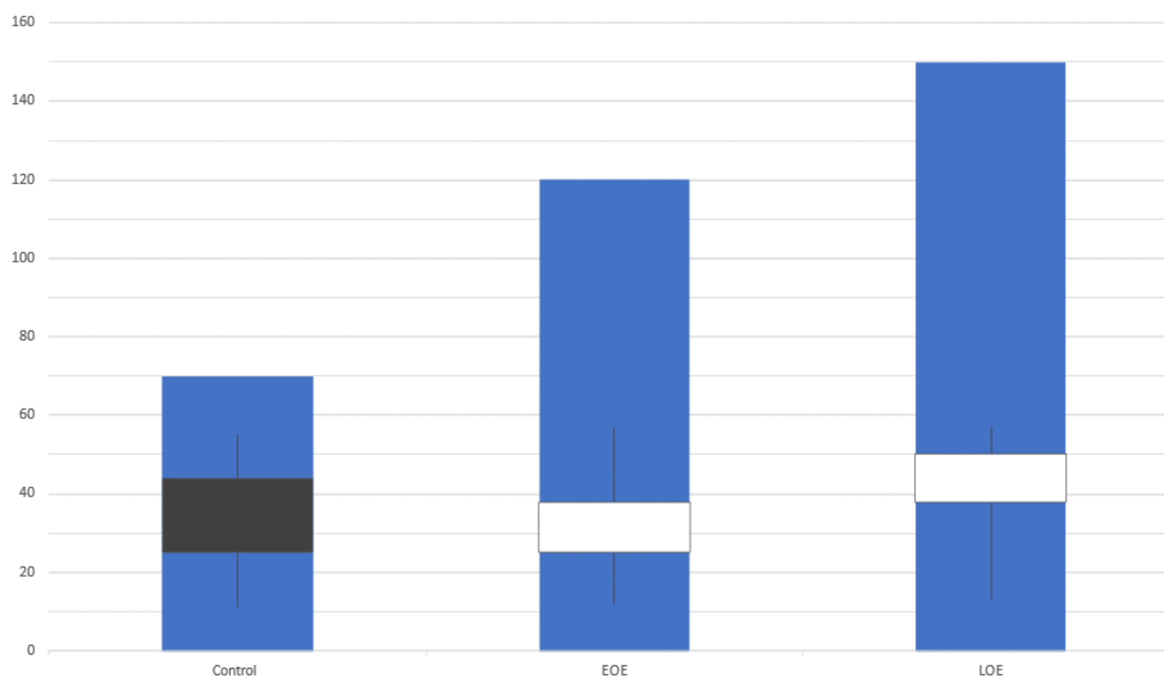

Figure 3. Glucose Uptake of two extract oil

Conclusion

Diabetes mellitus (DM), often known as sugar diabetes, is associated with high rates of mortality and disability worldwide. illnesses of the macrovascular system include cardiovascular myopathy and cerebrovascular illnesses, while microvascular disorders include retinopathy, neuropathy, and nephropathy. The reactive oxygen species (ROS) produced due to hyperglycemia considerably contribute to the development of the aforementioned problems by inducing lipid peroxidation and causing membrane damage. Cardiovascular problems are more common in patients with diabetes because of the prevalence of cardiovascular risk factors like obesity, hypertension, and dyslipidemia. Several different pathways must be addressed simultaneously for diabetic mellitus treatment to be successful. Sugar transport, insulin

signalling, insulin secretion, lipid management, inflammation, and oxidation are all examples of such processes. Weight gain and hypoglycemia are two undesirable effects of diabetes medication that can discourage patients from continuing treatment. By understanding the molecular structure of these compounds, new pharmacological agents that can be utilised to treat a variety of diseases can be created and developed. Monoterpenes, the primary component of essential oils, have been linked to numerous cellular and molecular functions. These functions may be responsible for the high therapeutic index observed in essential oils. Research and development on monoterpenes have taken on growing significance because of their abundant occurrence, wide range of biological action, and excellent safety profile. This article presented a summary of the many pharmacological classes available for the treatment of diabetes and their respective roles in the care of diabetic patients. We also collated the findings of a few chosen acyclic, monocyclic, and bicyclic monoterpenes thought to play a role in DM administration. Either cyclic or acyclic structures can exist among these monoterpenes. Differences in dose, duration, mode of drug administration, target tissue, and animal model make it impossible to generalise about the net antidiabetic impact of the three classes of monoterpenes. Experiments must be repeated with the same conditions as the original study to identify which type of monoterpenes (acyclic, monocyclic, or bicyclic) has the highest potential to exert an anti-diabetic effect. The concentration, the type of cell or tissue being targeted, etc., all play a role. Hydrocarbons like p-cymene and limonene are regarded to be the most active component, followed by alcohols such acyclic monoterpenes linalool and geraniol. It is speculated that aldehydes, including the acyclic monoterpene citral, have the most significant role. More research on the structure-activity relationship of monoterpenes, as well as structural modification, is needed to improve these compounds' medicinal benefits; this is a promising field of study. To fill in the blanks, they will need to be used in conjunction with other monoterpenes or natural compounds in the future. Currently, it is not known whether or if these compounds have therapeutic potential in treating hyperglycemia and dyslipidemia in human patients who have diabetes.

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