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SYBR-Green I fluorescence-based qRT-PCR of clove oil's anticancer, chemical characterization and antidiabetic activities

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ABSTRACT

Syzygium aromaticum is a valuable herbal plant in China, Europe, and Asia. It has been described as a powerful analgesic, anti-inflammatory, antioxidant, antifungal, and antibacterial agent. The goal of this work is to determine the phytoconstituents in clove oil derived from Syzygium aromaticum, a Myrtaceae family member. The GC-MS analysis of clove essential oil revealed 23 chemical compounds, including eugenol (58.65%), caryophyllene (6.60%), camphane (monoterpenes) (2.95%), Bisabolol (7.31%), tricosane (3.80%), eugenol acetate (3.35%), naphthalene (18.81%), and endo-borneol (19.98%). Certain organic acids such as benzoic acid (33.65%), acetic acid (9.30%), cinnamic acid (6.69%), and phenol (5.47 %). The anti-diabetic activity was evaluated utilizing an α-amylase suppression analysis. The anticancer effect of Cas 3 gene expression analysis by qRT-PCR was assessed in a human bone cancer cell line (MG-63). Clove oil inhibited the α -amylase enzyme concentration-dependently, with an IC50 value (IC50 167.24.46 µg/ml) equivalent to that of conventional acarbose (IC50 85.393.14 µg/ml). The results of real-time qRT-PCR revealed that following treatment with clove oil with an IC50 value of 33.6 µg/ml, the Cas 3 gene expression level in -actin and the human bone cancer cell line (MG-63) was expressed with a fold gene variation difference of 2.07. According to one study, clove oil could be utilized to treat diabetes and human bone cancer.

Keywords: *Syzygium aromaticum, Myrtaceate, Diabetes, Human bone cancer, GC-MS, Cas3 gene expression, α-amylase and β-actin*

INTRODUCTION

As the global cancer burden continues to rise, cancer poses a severe threat to global public health (Jemal, et al., 2011; Subasini, et al., 2013). Chemotherapy is still the greatest option for prolonging life and improving quality of life for individuals with late-stage cancer who have been diagnosed with the disease (Wyld and Reed, 2007). Despite these breakthroughs, existing medications have a modest impact on the five-year overall survival of patients with advanced disease, and drug resistance remains a key hurdle to the development of new treatments.

Significant use has been made of natural medicinal plants as a dependable supply of anticancer drugs. Up to 35 percent of anticancer drugs used worldwide are derived from plants, according to estimates (Saravanan, et al., 2011). The study of the active biomolecules found in medicinal plants continues to provide significant promise for the prevention and treatment of cancer (Huang, et al., 2010). Dried clove buds from Syzygium aromaticum L. have long been used in traditional Indian and Chinese medicine and as a spice. Cloves include a variety of sesquiterpenes and triterpenoids, including "oleanolic acid, stigmasterol, and campesterol, as well as eugenol, β-caryophyllene, humulene, chavicol, methyl salicylate, and eugenone, as well as flavonoids" (Park and Shin, 2005). Eugenol makes up 70–80 percent of the main parts of clove oil (Rani, et al., 2012; Uthirapathy, S., 2019). Most of the smell of clove oil comes from eugenol and volatile compounds (Uthirapathy, Subasini and Ahamad, Javed 2022; Sohilait, 2015). β-caryophyllene and eugenyl acetate are two more important parts of clove oil (Thenmozhi, et al., 2013). Other small minor parts include methyl salicylate, chavicol, α -copaene, α -amorphene, and caryophyllene oxide (Hossain, et al., 2014).

Clove, which is also called Syzygium aromaticum, is an Indonesian spice that comes from the Moluccas (Hadi, 2012; Subasini, et al., 2013). Indonesia produces an average of 80,000 tons of cloves each year, which is more than 60% of the world's cloves (Siagian, 2014; Thenmozhi and Subasini, 2016). Clove oil (CO) essential oil is well known for its dental analgesic qualities, and it has long been used as an antibacterial, carminative, and important food seasoning. Clove essential oil has a high therapeutic effectiveness. Chinese herbalists have used cloves and clove oil as traditional medicine for thousands of years. Although cloves are antibacterial, antifungal, antiviral, and bactericidal, it is unknown whether they are also anticancer (Jirovetz et al., 2006; Uthirapathy, Subasini and Tahsin, Amani., 2021; Atsumi et al., 2005; Javed Ahamad et al., 2019).

In this study, we investigated the molecular mechanisms and probable bioactive components underpinning clove oil's anticancer activity. Using human cancer cell lines, the effects of clove oil on cell proliferation, cell cycle distribution, and apoptosis were examined. qRT-PCR was used to explore the effects of clove oil (CO) on Cas 3 gene expression and to evaluate the efficacy of clove oil in treating diabetes by inhibiting the α -amylase enzyme.

MATERIALS AND METHODS

Plant materials and chemicals

The cloves were bought at the market in Erbil. A study of anticancer cell lines was done by the Research Institute of Biotechnology Pvt. Ltd. in Trichy. The MG-63 (Human bone cancer cell) cell line was bought at the NCCS Pune in India. Gibco (USA) provided the Rnase H-Reverse Transcriptase for Super-Script II. Forward and backward primers for the Cas 3 gene and -actin were bought from Xcelris Pvt. Ltd. (Table 1). The α -amylase enzyme was sent out by the Subra Scientific Company in Chennai, India. We bought a 96-well plate for growing cells from Tarson in India. All of the chemicals and solvents that were of analytical grade.

Preparation of essential clove oil from dried buds of cloves

In a Clevenger apparatus, 500 g of dried Syzygium aromaticum clove buds were hydro-distilated for 6 hours. The essential oil was collected in a graduated tube, dried over anhydrous sodium sulphate, and frozen at 4°C for later use to remove any leftover water.

GC-MS analysis and identification of phytochemical constituents of Clove oil

The volatile components were analyzed using a Shimadzu QP-2010 GC/MS system with a directly linked AB-Innowax 7031428 WCOT column (60 m x 0.25 mm x 0.25 m). GC-MS study of clove oil phytoconstituents (Uthirapathy, S et al. 2021, Subasini Uthirapathy et al. 2019).

Antidiabetic efficacy in vitro Assay for inhibiting α -amylase

The 3,5-dinitrosalicylic acid (DNSA) technique was used to perform the α -amylase inhibition experiment" (Miller GL, 1959). After completely diluting the mixture with 5 ml of distilled water and allowing it to cool to room temperature, the absorbance at 540 nm was measured using a UV-Visible spectrophotometer. Instead of the enzyme solution, a buffer solution was employed to measure absorbance. Acarbose (1000 µg/ml-31.25 µg/ml) was used to provide a positive control sample, and the reaction was carried out in the same manner as previously described with clove oil. To determine the percent inhibition of the -amylase inhibitory activity, calculations were performed. The IC50 values were calculated by plotting the percentage of α -amylase inhibition versus clove oil concentration.

Cas3 gene expression by quantitative qRT-PCR

MG-63 human bone cancer cells were grown in liquid media at 37°C in 5% CO2. Clove oil (CO) was evaluated on gene expression in MG-63 cells. "Trypsinized MG-63 cells were pooled in a 15-ml tube. Then, the cells were plated at 1×106 cells/ml cells/well (1 mL) into 6-well tissue culture plates in DMEM media with 10% FBS and 1% antibiotic solution for 24 hours at 37°C (Neah and Ujjwall, 2011). The wells were cleansed with sterile PBS, treated with 33.66 µg/ml of CO oil sample in serum-free DMEM media, and grown for 24 hours at 37°C in a humidified 5% CO2 incubator. MG-63 cell line total RNA was extracted using Trizol after incubation

RNA isolation

Total RNA was isolated using the manufacturer's TRIZOL method. The samples were centrifuged at 5000 rpm for 10 minutes in DEPC-treated tubes to get the cell pellet. The cell pellet (1X 107 cells) was lysed with 700 µl of TRIZOL. Lysate was collected in 1.5 ml tubes after forceful pipetting. Next, 300 µl of chloroform was added and vigorously agitated at room temperature for 5 min. The aqueous layer was separated by centrifugation for 20 min at 4oC at 12000 rpm. The 1.5-ml tube collected the aqueous layer. 700 µl of isopropanol was added for precipitated RNA. Precipitated RNA was pelleted for 20 min at 4oC at 12000 rpm by centrifugation. 70% ethanol sterilized the pellet. Air-dried RNA pellets were dissolved in 30 µl of double-distilled autoclaved water and stored at -80 °C until use. RNA quantity and quality were examined using a 1.5 percent agarose gel and a Labman UV-Vis Spectrometer.

Dnase treatment

RNA synthesis was DNA-free after Dnase. 1U of Dnase was added to 20 µl of reaction mixture. After 30–45 min at 37oC, 20 µM of 2 µl EGTA was added and incubated at 66oC for 10 min. Absolute ethanol (2V) and sodium acetate (1/10 V) were added and incubated at -20oC for 60 min. After 20 min at 12000 rpm at 4°C, the pellet was washed with 500 µl of 75% ethanol. The sample was air-dried, dissolved in 20 µl Milli-Q grade water, and stored until needed.

cDNA synthesis

To make cDNA from 1.5 µg of total RNA, reverse transcriptase (MMLV) was added. cDNA synthesis took 10 min at 25°C and 59 min at 42 °C. After reverse transcriptase inactivation and denaturation at 99oC for 5 sec, the cDNA/RNA hybrid was held at 4oC.

Micro-metastasis detection at the gene level

The cDNA template was used to detect apoptosis. ABI StepOne Plus (Applied Biosystems, CA, USA) SYBR®Green JumpStartTM Taq Ready MixTM for quantitative real-time PCR (qRT-PCR). qRT-PCR measured gene expression using relative quantification (2^-ΔΔCT) (Primer sequence Table 3). Beta actin normalized expression, and control cells calibrated.

RT-PCR condition

Initial melting temperature of 94°C for 3 minutes followed by 30 cycles of 30 sec at 94°C (Table 4). The annealing temperature was 51 °C for 30 sec, the extension temperature was 72 °C for 1 min, the final extension temperature was 72°C for 10 min, and the holding temperature was 4 °C.

Each expansion stage ended with real-time data. Results were presented using Ct. The relative quantity of target transcripts was determined using the comparative Ct technique (ΔΔCt), as per manufacturer directions. The 2−ΔΔCt method measured gene expression changes. Control PCR experiments without reverse transcription ensured genomic DNA did not contaminate total RNA.

Statistical Analysis

Data are presented as Mean±SD. The analysis uses GraphPad Software, Inc.'s Prism version 5. Statistical significance was $p < 0.05$.

RESULTS

Identification of the phytochemical components of clove oil by GC-MS analysis

The oil that was produced by hydro-distilling Syzygium aromaticum had the appearance of a pale-yellow liquid and smelled pleasantly fragrant. The ingredients of clove essential oil, together with their respective percentages and retention durations, are listed in Table 1. In the essential oil, the primary phytoconstituents identified include eugenol (58.65%), caryophyllene oxide (6.60 %), camphene (2.95%), eucalyptol (4.41%), bornanone (9.64%), and methanoazulene (11.5%). Endo-borneol was found to identify for 19.98% of the total, epicubenol for 6.31%, β -bisabolol for 7.31 %, carinol for 4.94%, tricosane for 3.80%, and organic acids including cinnamic acid and benzoic acid. Other minor components were also identified. There are a number of volatile chemicals that can be found in clove oils. Some of these substances include sesquiterpenes, phenyl propanoid, oxygenated sesquiterpenes, ester, ketone, and alkene. As a result, the volatile component might be able to help explain why cloves from various places have diverse flavors. The GCMS chromatogram of clove oil, which was isolated from clove flower buds, was presented in both Fig. 1 and Table 1.

Clove oil α -amylase inhibitory test

Isolated clove oil from clove buds exhibited a concentration-dependent α-amylase enzyme inhibitory activity that ranged from 59.05±3.56 to 1.66±1.94 at doses between 1000 and 31.25 µg/ml (Table 2). Acarbose, which was used as a positive standard, was able to block the enzyme beta-amylase at doses ranging from 44.89±3.28 to 27.85±2.02 for the clove oil sample. The α-amylase enzyme was inhibited by clove oil in a concentration-dependent manner, and the IC50 value (IC50 167.2±4.46 µg/ml) was discovered to be equivalent to that of standard acarbose (IC50 85.39±3.14µg/ml), respectively. Therefore, the facts that have been presented above suggest that clove oil may be very helpful in lowering the amount of starch that is absorbed by the body. Additionally, clove oil may be useful in the treatment of diabetes.

Cas3 gene expression by quantitative qRT-PCR

Clove oil's anticancer potential was tested in MG-63 (human bone cancer cells) cell lines, where it was found to suppress cell proliferation. For the MTT assay, the IC50 value was determined to be 33.66 µg/ml (data not shown). The capacity of clove oil to inhibit cell proliferation was demonstrated using a panel of human bone cancer cell lines. Cas 3 expression was analyzed in the human bone cancer cell line MG-63, and the results are presented in Table 5 and Fig (2a, b, and c). Positive expression of the Cas 3 protein was observed, in contrast to the control group. Cas 3 gene expression was normalized with plot amplification relative to an endogenous control, the -actin protein (Fig.2 d & e). Multiple quantitative real-time qRT-PCR cycles were used to normalize the Cas 3 gene (bcl-2) protein levels to the levels of -actin, as shown in Table 5. The results showed that Cas 3 protein was elevated with a value of 2.07 after treatment with clove oil.

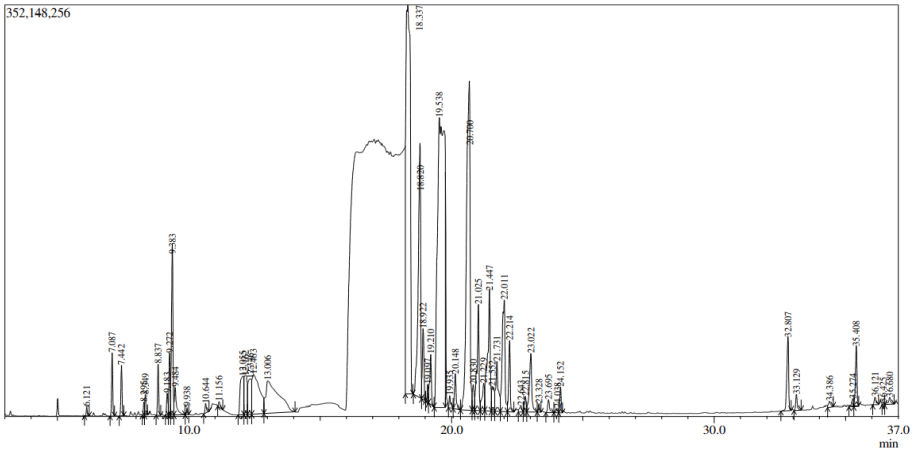

FIG 1: Clove oil - GCMS chromatogram from clove flower buds

TABLE 1: Clove oil's major and minor Phytoconstituents

S. No	Peak Name	Retention Time	% Area
1	1,3,7-OCTATRIENE, 3,7-DIMETHYL-, (E)-	7.087	3.01
2	Camphene	7.442	2.95
3	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETH	9.272	3.52
4	Eucalyptol	9.383	4.14
5	2-Heptanol, acetate	9.484	5.68
6	2-NONANONE	10.644	3.91
7	(+)-2-Bornanone	12.055	9.64
8	Endo-Borneol	12.463	19.98
8	BENZOIC ACID, 2-HYDROXY-, METHYL E	13.006	33.65
10	Eugenol	16.76	58.65
8	1H-3°,7-METHANOAZULENE, OCTAHYD	18.337	11.51
9	1,4-METHANOAZULENE, DECAHYDRO-4	18.820	9.07
10	Naphthalene, 1,2,3,5,6,8°-hexahydro-4,7-dimet	19.538	18.81
11	(-)-5-OXATRICYCLO[8.2.0.0(4,6)]DODECA	20.700	8.38
12	NAPHTHALENE, 1,2,3,5,6,7,8,8A-OCTAHY	21.447	4.61
13	Caryophyllene oxide	21.731	14.60
14	Alpha- Bisabolol	22.011	7.31
15	1-(2,3,4-TRIMETHOXYPHENYL)ETHANON	22.214	4.08
16	Junenol	21.552	7.0
17	B-methyl cinnamic acid, methyl ester	22.643	6.69
17	2,4-DIMETHYL-6-NITROPHENOL	22.815	4.26
19	BENZENE, 1-METHOXY-4-(2-PROPENYL)-	32.807	6.10
20	PHENOL, 2-METHOXY-4-(1-PROPENYL)-	35.408	5.47
21	Carinol	36.121	4.94
22	Tricosane	36.425	3.80
23	Benzofuran, 2,3-dihydro-2,2,5,6-tetramethyl-	36.680	6.50

TABLE 2: Clove oil has an inhibitory effect on the α-amylase enzyme

S. No	Conc.(µg/ml)	Acarbose (% inhibition)	Clove oil (% inhibition)
	1000	44.89±3.28	59.05±3.56
	500	46.26±3.77	56.61±3.18
	250	42.61±2.18	51.29±3.24
	125	39.87±2.05	13.54±2.34
	62.5	36.67±2.13	6.08±1.78
	31.25	27.85±2.02	1.66±1.94
	IC50	85.39±3.14	167.2±4.46

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TABLE 3: Primers used in Cas 3 gene expression study.

S.No	Primers Name	Oligo Sequence (5' to 3')
	CAS 3 F	5'-AGCAAACCTCAGGGAAACATT-3'
	CAS 3 R	5'-CTCAGAAGCACACAAACAAAACT-3'
	β -actin F	5'-ATCGTGCGTGACATTAAGGAGAAG-3'
	β- actin R	5'-AGGAAGGAAGGCTGGAAGAGTG-3'

TABLE 4: The working condition of qRT-PCR

Fragments	Initial denaturation	Number of cycles	Denaturation	Annealing	Final Extension
CAS 3	94°C -3 min	30	94°C – 30 sec	51°C – 30 sec	72°C -10 min
β- actin	94°C -3 min	30	94°C – 30 sec	51°C – 30 sec	72°C -10 min

TABLE 5: Gene expression of CAS 3 in bone cancer cells (MG-63) with treatment of clove oil

Sample	Reference gene – β- actin	Target gene- CAS 3	Δ Ct control	ΔΔ Ct (treatment) CAS 3	2^-ΔΔ Ct (treatment)	log 2 expression (fold variation change) CAS 3
			26.68	12.95	-13.73	2.07
β- actin	23.90	13.80				
Clove oil (CO)	26.68	12.95				

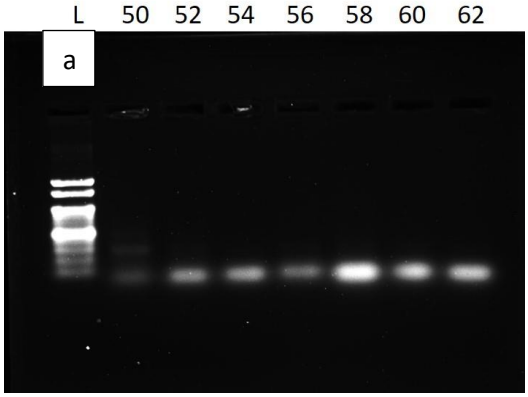

FIG. 2A: Gradient PCR analysis of Cas 3 gene expression at various degree temperatures

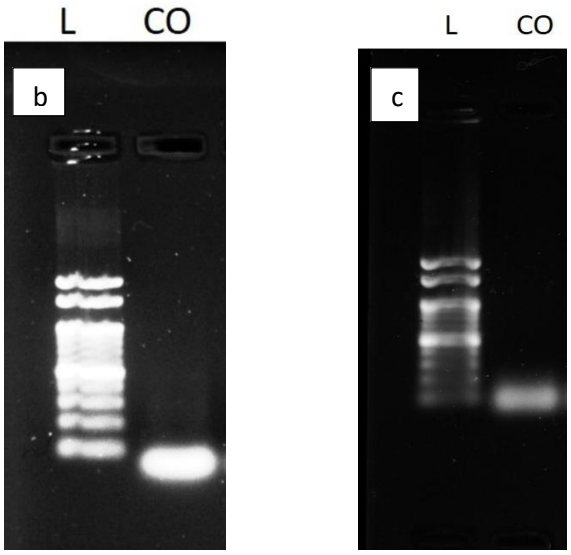

FIG. 2 (B & C) : Cas 3 gene expression and β-actin gene expression analysis of 33.66 µg/ml of CO

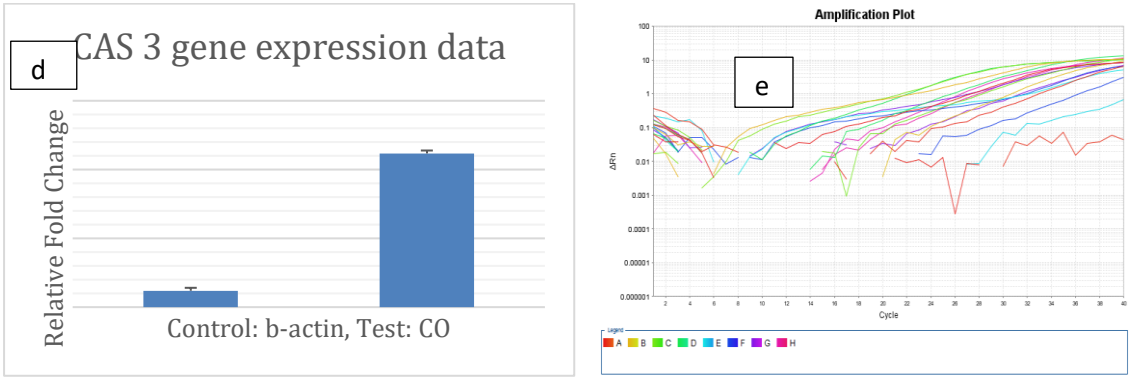

FIG. 2 D: Fold variation of Cas 3 gene expression in 33.66 µg/ml of CO treated samples and Fig. 2e: Amplification plot for case 3 gene.

DISCUSSION

Screening essential oils for antidiabetic action has become increasingly popular as researchers look for novel, effective treatments for the disease. However, the World Health Organization (WHO) suggests that a healthy diet and regular exercise can effectively control type II diabetes (WHO 2016). Therefore, one of the cheapest ways to manage the illness would be to get the urban populace to adopt a healthy lifestyle by eating more foods rich in antioxidants and reducing their sugar intake through the use of essential oils. Although flower bud and leaf extracts have demonstrated an antihyperglycemic activity in rat models, to the best of our knowledge this work is the first report on a detailed investigation of α-amylase inhibitory action. Some polyphenols, such as "gallic acid, ellagic acid, quercetin glucoside, an ellagic acid derivative, and numerous additional unknown phenolic components," have been characterized and detected in clove buds, as demonstrated by Atawodi et al. 2011.

Clove oil was used in this experiment, and while all of the main ingredients were the same, the proportions were different. Essential oil analysis revealed that eugenol, camphene, eucalyptol, bornanone, methanoazulene, and endo-borneol were among its most abundant phytoconstituents. Additionally, there were some trace chemical components that could only be found in one place. Unlike earlier published clove oils, the

local clove oil used in this study primarily included eugenol and caryophyllene (Tamilarasi, et al., 2000; Ahamad, et al., 2020; Jirovetz, et al., 2002; Thenmozhi, et al., 2016). The essential oil composition of a plant is often affected by the portion of the plant harvested and the time of year. However, "plant age, vegetation cycle, geographic location, mode of extraction," and methods of analysis are also important factors to consider when assessing the quality of an essential oil. "These factors may explain why this study's results differ from those of other studies" (Ferhat et al., 2006; Boussaada and Chemli, 2007).

Inhibiting carbohydrate-metabolizing enzymes such as α -amylase and -glucosidase is required to control postprandial hyperglycemia in diabetic patients. The α -amylase inhibitory investigation demonstrated that clove oil significantly inhibits the α -amylase enzyme based on the IC50 value of 167.2±4.46. Polysaccharides and disaccharides are degraded by enzymes called α -amylase and α -glucosidase, which are present in the GIT's brushing boundaries. Because they inhibit or delay the hydrolysis of 1,4-glycosidic bonds in other oligosaccharides, such as starch, into simple sugars such as maltose and maltotriose. These - amylase inhibitors are another term for these starch blockers (Javed Ahamad and Subasini Uthirapathy, 2021).

These enzymes reduce monosaccharide absorption, preventing the post-meal blood glucose spike. Blocking these enzymes controls postprandial hyperglycemia, the rise in blood sugar after eating. This effectively treats type 2 diabetes. Acarbose and miglitol, non-specific enzyme inhibitors that strongly block α-amylase and α-glucosidase, reduce polysaccharide metabolism. Thus, clove oil may be used to generate antidiabetic drugs by inhibiting the carbohydrate-breaking enzyme α-amylase (Tahsin, et al., 2022).

Since clove oil contains 80 percent eugenol, early research revealed that it inhibits cancer cell proliferation (Hussain et al., 2011). Quantitative qRT-PCR expression of the Cas 3 gene shows that the executioner Cas-3 can process 1000 proteins following activation (Crawford and Wells, 2011). Cleavage of these caspase substrates affects the activity of these proteins, which leads to apoptosis-related cellular changes. Cas 3 cleavage can also open routes. The DNAse caspase-activated nuclease (CAD) cleaves its inhibitor iCAD by caspase-3 to liberate chromatin between nucleosomes (Enari, et al., 1998). Caspase proteolysis inactivates vital physiological processes. ATP production, electron transport, and mitochondrial transmembrane potential are affected. when caspase breaks the p75 component of complex I chain during apoptosis (Ricci, et al., 2004; Uthirapathy, S., 2023). Some death systems produce proliferative signals to increase surrounding tissues. Signals depend on cell death type. Finally, death pathways are linked. Caspase activation enhances autophagic cell death and inhibits RIP-dependent necrosis. How a cell dies affects its neighbors and even the entire organism.

CONCLUSION

According to this investigation, the primary components of clove oil were discovered, though in varying proportions. A few small chemical components that were unique to the Iraqi region were also present. Clove oils contained monoterpenes, ester, ketone, sesquiterpenes, and organic acids, among other volatile substances. When using qRT-PCR techniques, clove oil from the buds demonstrates extraordinary -amylase inhibitory effect and significant anticancer efficacy. The creation of an anti-diabetic oral oil remedy made from clove buds will be aided by further research into the mode of action and safe and biologically effective application techniques.

REFERENCES

1. Ahamad, J., Uthirapathy, S., Ameen, M.S., Anwer, E.T., Hussain, F.H., Mir, S.R., 2020. Chemical composition and in vitro antidiabetic effects of Olea europaea Linn. (Olive). Current Bioactive Compounds, 16(8), pp. 1157-63.
2. Atawodi, S.E, Atawodi, J.C, Pfundstein, B, Spiegelhalder, B, Bartsch, H, Owen R., 2011. Assessment of the polyphenol components and in vitro antioxidant properties of Syzygium aromaticum (L.) Merr. & Perry. EJEAChe, 10(3), pp 1970– 1978.

3. Atsumi, S., Fujisawa, T., and Tonosaki, K. 2005. Toxicol. In Vitro 19, pp. 1025–1033.
4. Boussaada, O., Chemli, R., 2007. Seasonal variation of essential oil composition of Citrus Aurantium L. J. Essent. Oil-Bear. Plants, 10 (2), pp 109-120.
5. Crawford, E.D., and Wells, J.A., 2011. Caspase substrates and cellular remodeling. Annu Rev Biochem, 80, pp 1055–1087.
6. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., Nagata, S., 1998. A caspase-activated Dnase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature, 391, pp 43–50.
7. Ferhat, M.A., Meklati, B.Y., Smadja, J., Chemat, F., 2006. An improved microwave 133ydroalco apparatus for distillation of essential oils from orange peel. J. Chromato. A. 1112 (1-2), pp 121-126.
8. Hadi, S., 2012. Jurnal Bahan Alam Terbarukan 1, pp 25–30.
9. Hossain, M.A., S. R. Al Harbi, A., M. Weli, Q., Al-Riyami, and Al-Sabahi, J.N., 2014. Asian Pac. J. Trop. Dis, 4, pp 262–268.
10. Huang, X. H, Xiong, P.C, Xiong, C.M, Cai, Y.L, Wei, A.H, Wang, J.P., Liang, X.F, Ruan, J.L., 2010. In vitro and in vivo antitumor activity of Macrothelypteris torresiana and its acute/subacute oral toxicity. Phytomedicine, 17, pp 930–934.
11. Hussain, A., Brahmbhatt, K., Priyani, A., Ahmed, M., Rizvi, T.A., Sharma, C., 2011. Eugenol enhances the chemotherapeutic potential of gemcitabine and induces anticarcinogenic and anti-inflammatory activity in human cervical cancer cells. Cancer Biother. Radiopharm, 26, pp 519–527.
12. Javed Ahamad, Subasini Uthirapathy, Muath Sh. Mohammed Ameen & Esra T. Anwer 2019. Essential Oil Composition and Antidiabetic, Anticancer Activity of Rosmarinus officinalis L. Leaves from Erbil (Iraq). Journal of Essential Oil-Bearing Plants, (22)6, pp.1544-1553, DOI: 10.1080/0972060X.2019.1689179
13. Javed Ahamad., and Subasini Uthirapathy., 2021. Chemical Characterization and Antidiabetic Activity of Essential Oils from Pelargonium graveolens Leaves. ARO journal, http://dx.doi.org/10.14500/aro.10791
14. Jemal, A, Bray, F., Center, M. M., Ferlay, J, Ward, E and Forman D., 2011. Global cancer statistics. CA Cancer J. Clin, 61, pp 69–90.
15. Jirovetz, L., Buchbauer, G., Ngassoum M.B., and Geissler, M., 2002. J. Chromatogr. A 976, pp 265–275.
16. Jirovetz, L., et al., 2006. J. Agric. Food Chem. 54, pp 6303–6307
17. Kaskoos, R.A., Javed Ahamad, and Subasini Uthirapathy., 2021. Chemical Composition and Cytotoxic Activity of Pistacia atlantica var. kurdica Fruits. ARO-The Scientific Journal of Koya University, 9(2), pp. 91-95.
18. Miller GL., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem, 31, pp 426–428. Doi: 10.1021/ac60147a030.
19. Neah, L., Ujjwala, M.W., 2011. A unique method for isolation and solubilization of proteins after extraction of RNA from tumor tissue using trizol. J. Biomol. Tech. 22(1), pp 37-44.
20. Park, I.K, Shin, S.C., 2005. Fumigant activity of plant essential oils and components from garlic (Allium sativum) and clove bud (Eugenia caryophyllata) oils against the Japanese termite (Reticulitermes speratus Kolbe) J. Agric. Food Chem, 53, pp 4388–4392.
21. Raj, C.D., Shabi, M.M., Jipnomon, J., R. Dhevi, R., Gayathri, K., Subashini, U., Rajamanickam, G.V., 2012. Terminalia arjuna's antioxidant effect in isolated perfused kidney. Research in Pharmaceutical Sciences, 7(3), pp. 181-188.
22. Rani, B., Kachhawa, G.R., Yadav, R. K., Chauhan, V. and Raaz, M., 2012. Int. J. Res. Pharmacol. Pharmacother. 1, pp 230–233
23. Ricci, J.E., Munoz-Pinedo, C., Fitzgerald, P., Bailly-Maitre, B., Perkins, G.A., Yadava, N., Scheffler, I.E., Ellisman, M.H., Green, D.R., 2004. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. Cell, 117, pp 773–786.
24. Saravanan Subramaniam, Ramachandran Subramaniam, Suja Rajapandian, Subasini Uthrapathi, Victor Rajamanickam Gnanamanickam, Govinda Prasad Dubey., 2011. "Anti-Atherogenic Activity of Ethanolic Fraction of Terminalia arjuna Bark on Hypercholesterolemic Rabbits". Evidence-Based Complementary and Alternative Medicine, vol. 2011, Article ID 487916, 8 pages, 2011. https://doi.org/10.1093/ecam/neq003.
25. Schmitz, J.C, Chu, E., 2011. Effect of small interfering RNA 3'-end overhangs on chemosensitivity to thymidylate synthase inhibitors. Silence, 2(1), pp 88-93.
26. Siagian, V.J., 2014. Outlook Komoditi Cengkeh (Pusat Data dan Sistem Informasi Pertanian Kementrian Pertanian, Jakarta).
27. Sohilait, H.J., 2015. Science Journal of Chemisty 3, pp 95–99.

28. Subasini U, Sundaraganapathy R, Ananda Thangadurai S, Malathi R, Victor Rajamanickam G., 2011. Determination of Nutritive value for certain South Indian Indigenous Species. Int J Pharm & Ind FRes, 1(1), pp. 17-21.
29. Subasini U, Thenmozhi S, Sathyamurthy D, Vetriselvan S, Rajamanickam GV, et al. 2013. Pharmacognostic and phytochemical investigations of Dioscorea bulbifera L. Int J Pharm Life Sci 4, pp. 2693-2700
30. Subasini Uthirapathy, and Javed Ahamad, 2019. Phytochemical Analysis of different fractions of Terminalia Arjuna bark by GCMS, Int. Res. J. Pharm, 10(1), pp 42-48.
31. Tahsin, T., & Arif, A., & Bayrakdar, B, & Uthirapathy, S., 2022. In-Vitro Antioxidant Activity of Avocado Fruit Oil in Erbil, Kurdistan Region. Eurasian Journal of Science and Engineering, 8(3),298-307
32. Tamilarasi, C.T, Subasini, U, Kavimani, S, Jaykar, B., 2000. Phytochemical and Pharmacological Evaluation of Ampelocissus latifolia. Ancient Sci Life, 20(1), pp. 1-6
33. Thenmozhi S, Subasini U, Kameshwaran S, Dhanalakshmi M, Rajamanickam G.V., 2013. Morpho-anatomical and preliminary phytochemical studies of leaves of Gynandropsis pentaphylla Linn. Int J of Pharm & Life Sci, 4(7), pp. 2800-2809.
34. Thenmozhi S. and Subasini U., 2016. Isolation, characterization and in-vitro cytotoxic study of vitexin from Vitex pinnata Linn. leaves. IJRPP, 1, pp. 84–89.
35. Uthirapathy, S. (2023) "Cytostatic Effects of Avocado Oil Using Single-cell Gel Electrophoresis (Comet Assay): An Evaluation", Aro-The Scientific Journal Of Koya University, 11(1), pp. 16-21. doi: 10.14500/aro.11073.
36. Uthirapathy, S., 2019. Novel biomarkers of atherogenic diet induced dyslipidemia and metabolic syndrome suppressed by Terminalia arjuna. Int J Pharma Sci Res, 10, pp.2528–2536. https://doi.org/10.13040/IJPSR.0975-8232.10(5).2528-36.
37. Uthirapathy, S., and Tahir, T. F., 2021 "Withania Somnifera: Correlation of Phytoconstituents with Hypolipidemic and Cardioprotective Activities", Aro-The Scientific Journal Of Koya University, 9(2), pp. 15-21. doi: 10.14500/aro.10844.
38. Uthirapathy, Subasini and Ahamad, Javed., 2022. *Toxicity profiles of the Hydroalcoholic seed extract of Psoralea Corylifolia L Fabaceae in Wistar rats.* Academic Journal of Health Sciencies Medicina balear, 37 (3), pp. 120-126
39. Uthirapathy, Subasini and Tahsin, Amani., 2021. Evaluation of Genotoxic effects of a Hydro-alcoholic extract of flowers of Nargis *(Narcissus Tazetta L.)*. Eurasian Journal of Science & Engineering, 7 (2). Pp. 39-48.
40. WHO Fact Sheet Fact sheet. 2015. N°312 http://www.who.int/mediacentre/factsheets/fs312. Accessed 24 Jan 2016
41. Wyld, L, Reed, M., 2007. The role of surgery in the management of older women with breast cancer. Eur. J. Cancer, 43, pp 2253–2263.
42. Siagian, V.J., 2014. Outlook Komoditi Cengkeh (Pusat Data dan Sistem Informasi Pertanian Kementrian Pertanian, Jakarta).
43. Sohilait, H.J., 2015. Science Journal of Chemisty 3, pp 95–99.