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Anti-diabetic study and its Quantitative Real-time PCR analysis of the INSR gene in Silvernanoparticle extract of *Chamaecostuscuspidatus*

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Abstract

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Manufacturing metal oxide nanoparticles for chemical and biomedical applications is made possible by green synthesis, an eco-friendly approach.This investigation examined how silver nanoparticle leaf extract affected gene expression and its ability to treat diabetes.In this study, the Anti-diabetic study was evaluated by following the Glucose utilization control method. Furthermore, the study continues with Quantitative Real Time-PCR Analysis of the INSR Gene.The analysis's findings showed that, when used as a control, metformin exhibits a glucose percentage of 169.26, whereas silver nanoparticle extracts exhibit a percentage

control of 169.53.HepG2 cells treated with AgNPs*C.cuspidtus* showed upregulated INSR gene expression, going from 1-fold in control cells to 1.51-fold in treated cells. The relative gene expression of the control is typically set to 1 when compared to the sample.The study shows that glucose absorption in AgNPs extract is slightly higher than in methanolic

extract.As a result, there is a pressing mandate to protect this species and investigate its biological applications to achieve more significant and worthwhile outcomes.

Keywords: INSR genes, RT-PCR, Anti-diabetic, Silver nanoparticles, Hep G2 Cells

Introduction

The Costaceae family, thought to contain some of the most significant medicinal plants, includes *Chamaecostuscuspidatus*, also known as the insulin plant.This plant has many secondary metabolites and bioactive components dispersed across its various portions, and the leaves showed high levels of protein, iron, and antioxidants such as ascorbic acid, -tocopherol, -carotene, terpenoids, steroids, and flavonoids (Hedge et al., 2014).Another study reported that methanolic extract had the highest concentration of phytochemicals, including sugars, triterpenoids, proteins, alkaloids, tannins, saponins, and flavonoids.

Moreover, the exclusive focus of this study, however, is on silver nanoparticles, a recent innovation in science.Metal nanoparticles can be created via a wide variety of physical, chemical, biological, and hybrid processes. Physical and chemical processes typically produce low yields, are hazardous, and are not eco-friendly.Compared to physical, chemical, and microbiological methods of nanoparticle manufacturing, green methods are more advantageous in terms of the environment, cost, energy efficiency, and compatibility with pharmaceuticals (Jyoti et al., 2016).Additionally, nanoparticles made from plant materials or extracts are more biocompatible because they contain functional proteins that actively decrease metal ions (Suresh et al., 2018).

The plant has anti-diabetic characteristics; thus,an anti-diabetic study was also conducted. The technique used in this study was the glucose utilization method using Hep G2 cells and metformin, which is regarded as a positive control in this investigation. According

to Motloung et al. (2020), loss of pancreatic insulin production, mainly brought on by autoimmune pancreatic ß-cell destruction, is a hallmark of type 1 diabetes. Type 2 diabetes (T2D), the most common type of diabetes (approximately 90% of cases), is brought on by partial ß-cell malfunction and target tissue insulin sensitivity. Furthermore, the studies reveal that Insulin resistance in T2D causes impaired hepatic glucose production and reduced glucose absorption in peripheral tissues, contributing to chronic hyperglycemia(Motloung et al., 2020).

Following this, the same extract was used to concentrate on gene expression using the INSR gene. By altering the binding of transcription factors to insulin-response regions or controlling their transcriptional activity, insulin affects the transcription of genes.Many insulin-signalling cascades have been determined to mediate the insulin effect on gene transcription (Mounier& Posner., 2006).The study focuses on rt PCR test quantitative analysis at varying stages.This study focused on the plant leaf extract containing silver nanoparticles to investigate the anti-diabetic properties and gene expression.

Materials & Methods

Preparation of plant extract

10gm of fresh *C.cuspidatus* leaves was cut and rinsed well under running water to eliminate surface impurities and debris. Absolute alcohol was then used to sterilize and disinfect it thoroughly. The leaves were sliced into small pieces, placed in a beaker with 100ml of distilled water, and then heated for about 20 minutes. After the temperature of the plant extract comes to room temperature, a filtrate is extracted by filtering it using No. 1 Whatman filter paper. Refrigerate the filtrate at 4 degrees Celsius until the silver nanoparticles are synthesized.

Preparation of Silver Nanoparticles

A molar standard aqueous solution of Silver nitrate ($AgNO_3$) solution was prepared by dissolving 17 gm of silver nitrate in 1 litre of deionized water, which was utilized to synthesize nanoparticles. The next step involved the addition of 5 ml of plant extract of *C.cuspidatus* to 45 ml of standard 0.1M $AgNO_3$ solution in a 100 ml Erlenmeyer flask. The entire process was conducted at room temperature to facilitate the formation of silver nanoparticles. The shift in colour from white to dark brown could be considered a positive indication of the formation of silver nanoparticles. The mixture is centrifuged at 10,000rpm and rinsed with 70% alcohol to extract the nanoparticles from the reaction medium.

Antidiabteic activity

HepG2 cells were cultured in a DMEM medium containing4.5 g/L D-glucose, 10% heat-inactivated FBS, and 5% CO2 at 37°C. Before being planted into 96-well plates with 5000 cells per well and six blank wells for three days of growth, the cells were trypsinized and counted using a trypan blue assay.After three days of seeding, the extracts were added in triplicates at concentrations of 5, 10, 25, 50, and 100 l. The used culture media was removed after 48 hours of incubation and replaced with a 25-litre batch of incubation buffer (DMEM diluted with PBS, 0.1 percent BSA, and 8 mm glucose), which was then incubated for an additional 3 hours at 37 degrees Celsius. 0.1 µg/ml (Motloung et al., 2020).

Metformin served as the positive control, and incubation buffer without extract was the negative control for the group that was not given any medication. Following the incubation period, 10 l of the incubation media from each well was collected and transferred to a fresh 96-well plate. 200 l of the glucose oxidase reagent was then added to the plate to determine the amount of glucose in the medium.After 15 minutes of incubation at 37 C, the absorbance was measured at 492 nm using a Multiscan plate reader. The difference between the cell-free

and cell-containing wells was employed to calculate the amount of glucose utilized. The percentage of glucose consumption was evaluated in comparison to the untreated controls.

Quantitative Real Time-PCR Analysis of INSR Gene (Stuart et al., 2003)

Sample preparation

Prepare *C.cuspidatus* leaf extract with fresh silver nanoparticles for the synthesis process.

Primer synthesis

S.No.	Gene	Primers
1	INSR	F-AGGCTCCCGTCTCTTCTTCAA R-GACATCCCCACATTCCTCGTT
2	GAPDH	F-ATGGCATTCCGTGTTCCTAC R-CCTTCAACTTGCCCTCTGAC

List of primers

The sequences were then sent to a 50nm Scale with HPLC Purification for synthesis after being chosen. After acquiring the synthesis, primers were generated to a stock concentration of 10 pMolar and then to a working concentration of 10 pM before being used in the Realtime-PCR.

RNA isolation

Cells were scraped after being exposed to AuNP for 24 hours, and total RNA was then extracted using TRIzol by Takara Bio Inc. A 1 ml Tri reagent solution was used to scrape the cells, and any insoluble material was removed by centrifuging the sample at 12,000 g for 20 minutes at 4 °C.0.2 mL of chloroform was added aggressively mixed into the clear lysate.

The phases were separated by centrifugation at 12,000 g for 20 min at 4 °C.The upper aqueous layer containing the RNA was collected in a fresh tube, and the RNA was precipitated with isopropanol (0.5 ml) and centrifuged at 12,000 g for 10 minutes at 4 °C.Pellet was washed in 75% ethanol, resuspended in RNase-free water, and purified using the RNeasy Mini Kit (Qiagen). RNA samples were kept at -80 °C until needed.

cDNA Synthesis

The Takara Prime Script 1st strand cDNA synthesis kit (Cat No 6110A) was used to generate cDNA from extracted RNA.The experiment was carried out in a thermocycler utilizing a 2x reverse transcription (R.T.) master mix containing 10X R.T. buffer, 25X dNTP mix, 10X R.T. random primers& 4 µg of RNA was reverse transcribed using 2xreverse transcription (R.T.). Multiscribe reverse transcriptase, RNase inhibitor, and nuclease-free water (Bio-Rad)&The following were the reaction conditions: Initial R.T. for 5 minutes at 65°C, then 50°C for 60, followed by reverse transcriptase inactivation at 95°C for 5 minutes.

Real-time PCR

Each reaction employed 25 ng of cDNA, 10 pmolar forward and reversed primers and a final reaction volume of 20 µl for Real-Time PCR. SYBER green used the Qiagen Rotor-Gene Q real-time PCR to investigate gene expression.The cycle at which each reaction achieved the set threshold (C.T.) was determined, and the detection of product formation was set in the centre of the linear section of PCR amplification. The CT values were used to compute the relative change in mRNA expression.Levels were normalized to GAPDH mRNA, the housekeeping gene, and presented as fold changes over controls.

Initial denaturation at 94^{0}C for 2 minutes was followed by 35 cycles of denaturation for 30 seconds at 94^{0}C, annealing at 60^{0}C, and extension at 72^{0}C. T. The final extension

temperature will be 72^{0}C for 5 minutes. All reactions, including no-template controls, were performed in triplicate. The relative gene expression levels were calculated and tabulated.Upregulation of INSR gene expression was observed from 1-fold in control cells to 1.51 folds in AgNPs*C.cuspidtus* treated cells of HepG2.

Results

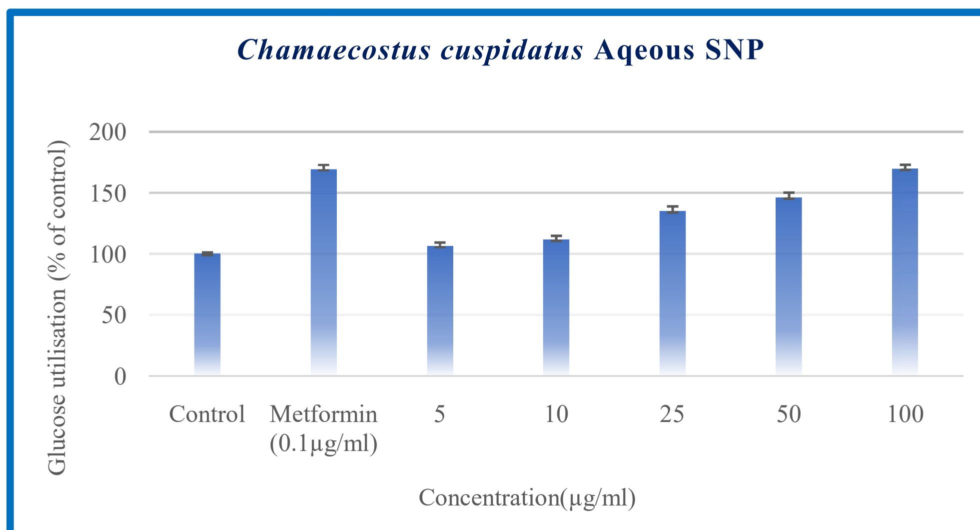
The results of the anti-diabetic assay of *Chamaecostuscuspidatus* leaf extracts in silver nanoparticles have been investigated, and the method used in the investigation is glucose uptake control using metformin and a control sample. A quantitative real-time PCR analysis test was also studied, considering INSR genes.

The findings of this study on anti-diabetic assays showed that the control at 0.612 nm O.D. shows 100% glucose utilization, whereas the O.D. of 0.1 g/ml metformin was determined and indicated 1.035 nm and 169.26 glucose utilization.

Extract (µg/ml)	**OD**	**Glucose Utilization(% Control**
Control	0.612	100
Metformin (0.1µg/ml)	1.035	169.26
5	0.65	106.37
10	0.682	111.52
25	0.825	134.86

50	0.894	146.18
100	1.037	169.53

Table: In vitro Anti-diabetic activity of *Chamaecostuscuspidatus* Aqueous SNP



In vitro Anti-diabetic activity of *Chamaecostuscuspidatus* Aqueous SNP

Quantitative analysis of Real-time PCR test

The focus of this study is the quantitative analysis of a real-time PCR test in an AgNPs leaf extract of *Chamaecostuscuspidatus*. Two genes were chosen, including the gene of interest, INSR genes, and the housekeeping gene GADPH, and two primers were used to synthesize and isolate the RNA. These primers undergo sequencing, and HPLC is purified and ready to use in quantitative analysis. The cells are treated with AgNPs, and total RNA is isolated using a TRIzol reagent. After that, cDNA synthesis is carried out and preceded byoneµg of total RNA isolated.The A260/280 ratio of the Hepatic G2 cell control is 1.77, while the ratio of the extract is 1.73. The ratio indicates the purity of DNA and RNA; if it is less than 1.8, the extract contains contaminants like proteins and phenols that can be treated further by lowering the Ph values.The concentration of cDNA is concluded by measuring the

absorbance at 260 nm in a biophotometer against a blank. The O.D. of the sample and concentration at 260 nm was determined.

S. No	Sample Name	A260/280	cDNA Concentration(μg/ml)
1	AgNPs extract	1.73	452.8
2	HepG2 Cell control	1.77	463.2

Concentration of cDNA

Following the cDNA synthesis, reverse and forward transcriptase primers were used to investigate real-time PCR. SYBER green used Qiagen Rotor-Gene Q real-time PCR to investigate gene expression, and these reactions were run in triplicates as control and treatment samples. The relative gene expression levels were computed and tabulated.

As the fold change is greater than 1, the gene of interest is elevated if the ΔΔCt is negative. If the ΔΔCt value is positive, the gene is downregulated, and the fold change is less than one. Upregulation of INSR gene expression was observed from 1-fold in control cells to 1.51 folds in AgNPs*C.cuspidtus* treated cells of HepG2. Compared to the sample, the relative gene expression of the control is generally set to 1.

Samples	GOI	GAPDH	ΔCT	ΔΔCT	2^-(ΔΔC.T.)	
Control 1	35.31	31.14	4.17	-0.75333	1.69	
Control 2	36.53	30.27	6.26	1.336667	0.40	
Control 3	35.25	30.91	4.34	-0.58333	1.50	**Avg Cl: 1.19**
Treated 1	31.64	27.54	4.1	-0.82333	1.77	
Treated 2	32.06	27.11	4.95	0.026667	0.98	
Treated 3	31.12	27.42	3.7	-1.22333	2.33	**Avg td: 1.70**

			4.586667	**-0.33667**	**1.444326**	

C.T. values of samples

One of the most crucial indicators of a qPCR assay's success is its PCR efficiency, an essential metric for quantitative analysis when fold changes are determined. In order toproperly use PCR efficiency in qPCR analysis, it must be estimated with high precision (Svec et al., 2015).The generation of a series of samples with regulated relative amounts of a targeted template is required to estimate PCR efficiency using a standard curve.

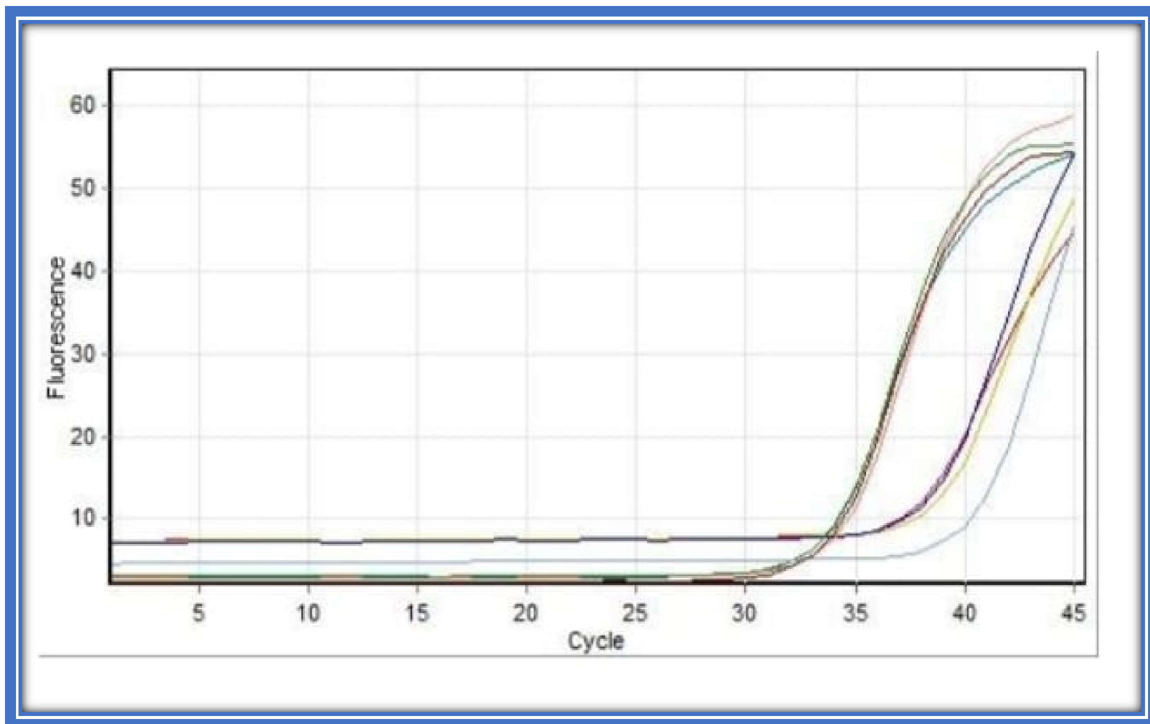
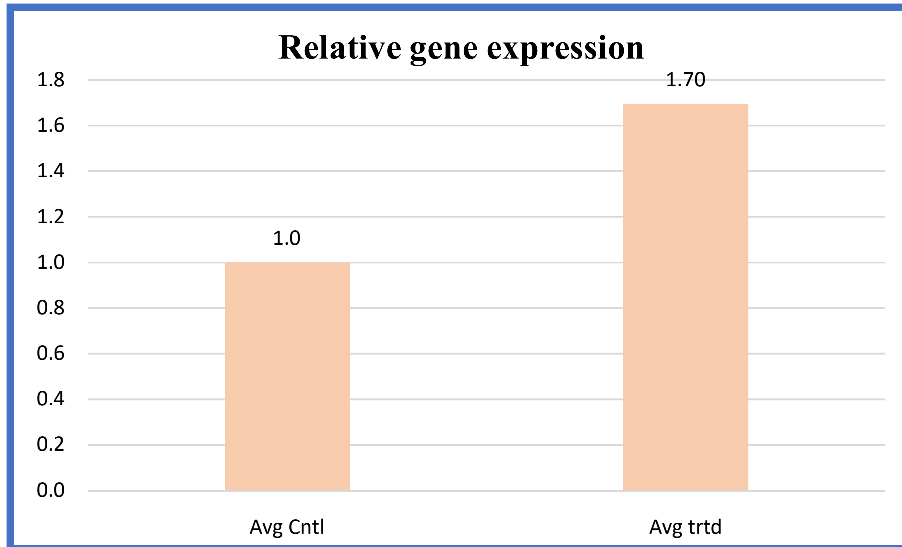


Fig-53: PCR efficacy



Relative Gene Expression of INSR gene in HepG2 Cells

Sample	ΔCt	ΔΔCt	2^-(ΔΔCt)
AgNPs*C.cuspidatus*	**4.586667**	**-0.33667**	**1.444326**

ΔΔCt Values of Samples

Table24: Relative Gene Expression of INSR

	Control		Samples	SEM
AgNPs*(C.cuspidatus)*	1		**1.444326**	**0.37612**

Discussion & Conclusion

Recent developments in anti-diabetic medication discovery indicate that mineral supplements may be attractive candidates for creating effective anti-diabetic drugs.Because of the complexity of accompanying consequences and its global development, diabetes has emerged as one of the most severe metabolic disorders in the world (Odeyemi& Dewar, 2019).Metformin, taken as a standard, has a glucose consumption of 169.26 at 0.1 g/ml, according to the present diabetes study. At a 5 g/ml concentration, a methanolic extract demonstrates 105.62 percent glucose utilization, but an AgNPs extract indicates 106.37 percent glucose utilization at the same concentration as a methanolic extract. As a result, the study shows that glucose absorption in AgNPs extract is slightly higher than in methanolic extract.

Quantitative PCR (qPCR), also known as real-time or quantitative real-time PCR, is a PCR-based technique that combines the amplification of a target DNA sequence to determine the DNA species' concentration in a reaction (Lorsch &Dymond, 2013).The comparative Ct technique is a mathematical model for calculating gene expression changes as a relative fold variation between an experimental and calibrator sample (Wong & Medrano, 2005).The current study focuses on RNA isolation, cDNA synthesis and real-time PCR. INSR genes are employed in this study, providing instructions for producing an insulin receptor protein found in various cells.Since the anti-diabetic assessment demonstrates efficient glucose uptake in AgNPs extract, a real-time PCR investigation focused on the AgNPs extract revealed upregulation of relative gene expression. INSR gene expression was upregulated 1.51 folds in AgNPsC.cuspidtus treated HepG2 cells, compared to 1-fold in control cells.

Chamaecostuscuspidatus is an insulin plant of the Costaceae family, and the leaves can be pulverized to suppress sugar levels. That is why glucose uptake is more effective in both extracts, although silver nanoparticles are more environmentally friendly and easier to produce.The real-time PCR quantitative analysis test, shows that the AgNPs leaf extract can be used in therapeutics and medication formulations.Furthermore, the AgNPs extract opens up new pathways for researchers to investigate the chemicals that cause these therapeutic effects and the mechanism of action.More research is needed to investigate the mechanism of action of chemicals and the standardization of herbal pharmaceuticals using models, which would help developvarious anti-diabetic drugs at a reasonable cost.

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