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ACKNOWLEDGEMENT

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Wastewater Treatment Using Biotechnological Approach

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Hazardous chemicals are forming at an accelerating rate due to a growing global population, necessitating ecological response. Wastewater management and treatment is a costly operation that needs the right technological integration to make it more practical and affordable. Algae are highly sought-after as potential feedstocks for a variety of applications, such as sustainability of environmental, the generation of bioproducts with high-worth, and the manufacturing of biofuels. One technique to reduce wastewater contamination is microalgae bioremediation. The demand for effective greenhouse gas reduction, wastewater treatment biomass reuse and nutrient recovery, has spurred a major concern in the microalgae use of wastewater treatment. Algal biomass can also be used to produce high-value bioproducts and bioenergy. Researchers from all over the world have investigated the use of microalgae for food additives, biofuels, and the production of bioactive and medicinal compounds. The commercial use of algae is now prohibited by technological and financial constraints, and efficient downstream processes are required to lower production costs. Therefore, using microalgae for both biofuel generation and wastewater treatment simultaneously could be a cost-effective way to solve both problems [1]. A different method is to use native bacteriophages as markers to detect the existence of enteric viruses of human, taking into account the bacteriophages. Native bacteriophages in particular share many characteristics with enteric viruses of human, including composition, size, structure, and replication-related elements. For instance, bacteriophages e.g FRNA have an isoelectric point (IEP) of 3.9and dimensions of about 25 nm and, which are related to those of the enterovirus (human) and hepatitis A virus (both of which have sizes of 22–30 nm and IEPs of 4.0–6.4). (IEP 2.8, 27e28 nm). Importantly, the technology used in bacteriophage assays is the quickest and least expensive for identifying human enteric viruses. Researchers are actively striving to quantitatively examine the link between human enteric viruses and native bacteriophages in order to determine the best indicator and improve prediction accuracy. Nevertheless, contrasting assumptions have emerged from the published investigations. The elimination of human pathogenic waterborne viruses, particularly enteric viruses of human, is a crucial factor to consider when assessing the efficiency of membrane treatment in the production of wastewater and drinking water [2]. An effective method for treating spent water that uses little energy is deammonification, which combines partial nitridation and anaerobic ammonium oxidation. Since the 1990s, when Anammox bacteria were first found, numerous full-scale side stream deammonification units handling high-ammonia used water have been operating successfully. However, there haven't been many reports of this method being utilized extensively to treat municipal waste water with low ammonia concentrations [3].

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Original Article

VEGF Gene Polymorphism Among Diabetes Mellitus and Diabetic Retinopathy

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a major angiogenic factor and a prime regulator of endothelial cell proliferation. VEGF gene is located on chromosome 6 (6p21.3). **Objectives:** To determine whether deletion at -2549 position of promoter region of the VEGF gene exert influence on the development of diabetic retinopathy. **Methods:** Diseased and control subjects were selected for blood sampling. The blood samples (n=50) was taken from diabetic retinopathy patients and blood samples(n=50) was taken from type 2 diabetes mellitus patients as control group. After DNA extraction Polymerase Chain Reaction was performed to amplify the VEGF gene and sequencing was commercially done for molecular analysis of VEGF gene. **Results:** The molecular analysis confirmed that deletion at -2549 position of the promoter region of VEGF significantly greater in DR group. 2-11 % deletion was examined at -2549 position of promoter region of VEGF. The DD genotype was responsible for the development of DR. **Conclusions:** This study indicates that DD gene mutation and D allele is an autonomous hazard aspect for the advancement and progression of retinopathy in people with type 2 diabetes, additionally different issues for example diabetic age and family ancestry of diabetes assumes key function in the advancement of retinopathy in diabetic patients.

INTRODUCTION

Retinopathy is a severe disease of the retina of eye. For some cases, it is the primary reason of impaired vision. Among non-diabetic patients, eye disorders related with retinopathy contains visual obstruction such as retinal telangiectasia and microaneurysms in the retina. Chronic disorders linked to retinopathy in patients with no diabetes contains chronic hypertension and general disorders. Retinopathic characteristics namely microaneurysms, dot and blot leakage and spots of cotton wool may be dependable on the identification of DR in certain people above the age of 40 without DM [1]. PDR is the most prevalent vision threatening abrasion mostly in people with type 1 diabetes. But, DME causes much of the blindness suffered by diabetes people since it is the main reason of the blindness in extremely widespread T2D and is typically exists in PDR type 2 diabetes patients [2, 3]. Numerous risk factors have previously been reported for macular edema, for example period of diabetes, impaired glycemic regulation and diabetic nephropathy. Type 2 diabetes symptoms namely increased age, hypertriglyceridemia and high blood pressure can also raise the threat of macular edema [4]. Angiogenesis of retina due to ischemia and too much discharge of extracellular matrix causes the development of fibrovascular tissues in PDR at the vitreoretinal boundary. The development of fibrovascular membrane, which consists of new blood vessles, αsmooth muscle actin (α-SMA)-expressing myofibroblasts and leukocytes frequently causes blindness due to detachment of retina. Hypoxia appears to cause neovascularization by angiogenic factor upregulation in PDR [5, 6]. Prematurity retinopathy (ROP) is an irregular retinal neovascularization of visual condition that infects premature babies. The ailment can assist the retina dispassion and is one of the major reason of everlasting vision loss in preterm infants

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[7]. DR is a multifaceted disorder in addition to prolonged inflammation and oxidative stress induced by leukocytes, some studies report the function of proinflammatory cytokines and angiogenesis stimulating molecules in the pathological process of the disease [8].

METHODS

For this study, a total of 100 people (n=100) were chosen and evenly divided into two groups: control and sick. People with type 2 diabetes (n=50) were termed the control group, whereas those with diabetic retinopathy (DR) (n=50) were considered the sick group. All subjects' blood samples were obtained in labelled falcon tubes containing (200l) 0.5M EDTA solution. All of the falcon tubes were vortexed to mix the blood and EDTA solution, and the tubes were then kept in a freezer at -20oC. To separate DNA from blood tests, an organic standard chloroform and ethanol precipitation method was used, followed by cell disruption, protein destruction via proteinase K, protein deposition using soaking NaCl, and DNA accumulation via isopropanol. Gel electrophoresis confirmed the extracted DNA. By using 8g agarose, 0.8% agarose gel was prepared for this objective. In 100ml of 1X TBE 0.8 g agarose was included during gel preparation, then heated on hot plate till agarose was totally broken down in buffer and a clear solution was found. 4-5µl of EtBr was included in it and mixed it appropriately. Under the gel doc apparatus, gel was visualized. Quantification of DNA was completed in this way.Oligonucleotide primers were used for the amplification of needed gene fragment or polymerase chain reaction (PCR). A particular set of forward and reverse primers were used for VEGF gene Forward: 5′ GCTGAGAGTGGGGCTGACTAGGTA-3′(24bp) and Reverse: 5′ GTTTCTGACCTGGCTATTTCCAGG-3′ (24bp). Starting denaturation for 5 minutes at 95^{0}C, 35 cycles each for 30 seconds at 94^{0}C, annealing at 61.7^{0}C for 1 minute and 20 seconds then extension at 72^{0}C for 1 minute and final extension at 72^{0}C for 10 minutes. PCR products optimization was verified with 2% agarose gel. Each PCR product (3µl) was combined with 2µl of 6X loading dye. The loaded DNA samples were run for 35 minutes at 120V on 2% gel versus 4µl of Marker (1kb DNA ladder). Gel was then shifted to gel doc for the visualization of PCR products. The 18 bp I/D fragment was further confirmed by commercial sequencing to confirm the size of I/D, its sequence and I/D region in the population of Lahore.

RESULTS

The present research was intended to discover the relationship of diabetic retinopathy (DR) with VEGF gene polymorphism. Within 100 study subjects, 50 subjects having DR considered as unhealthy group and 50 were control having DM. The PCR products were sent for commercially sequencing. Following are the sequences of diseased and control samples. These sequences then further blast on NCBI to check the alignments. Following are the alignments of diseased and control subjects. These sequences showed 2-11% mutation (deletion) at -2549 position of the promoter region of the VEGF gene in diabetic retinopathy subjects (Figure 1-Figure 04).

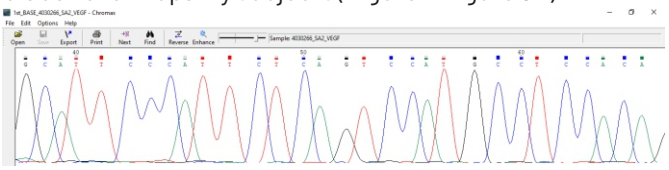

Figure 1: DNA Sequencing chromatogram demonstrating deletion (mutation) at -2549 promoter region of VEGF gene in diseased group (DR)

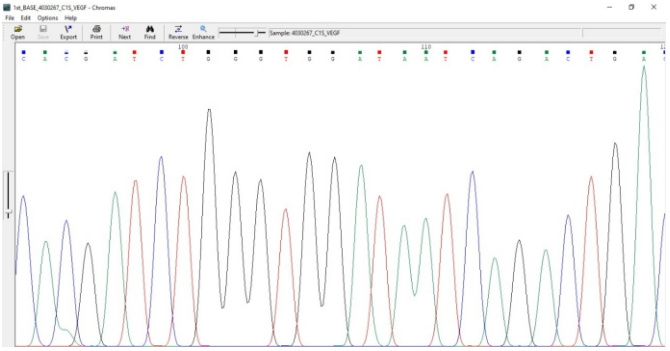

Figure 2: DNA Sequencing chromatogram demonstrating Insertion (mutation) at -2549 promoter region of VEGF gene in Control group (DM)

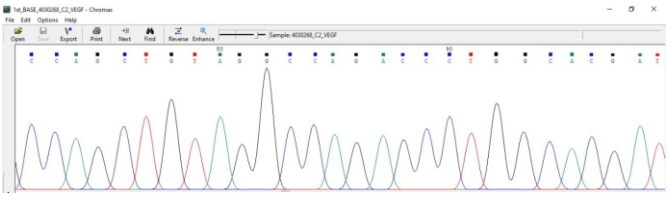

Figure 3: DNA Sequencing chromatogram demonstrating Insertion (mutation) at -2549 promoter region of VEGF gene in Control group (DM)

Vascular endothelial growth factor gene VEGF I/D mutation are seen in figure 4. The band for DD gene mutation is seen at 211bp in diabetic retinopathy subjects. Huge contrasts were seen in diabetic retinopathy and diabetic people.

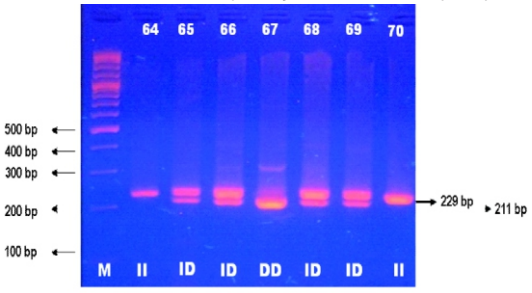

Figure 4: PCR amplification products of VEGF I/D at -2549 position of promoter region

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DISCUSSION

As per the past examinations, vascular endothelial growth factor has solid relationship with diabetic retinopathy. Vascular endothelial growth factor has double function in functioning as just in pathology of retina. Regularly it is discharged in least quantity from cells of retina yet, when high blood sugar level problems because of diabetes then oxidative pressure and irritation in endothelial cells results in vascular breakdown and deterioration of neuroglia [9]. In the present study the potential association of the 18bp fragment (I/D) polymorphism of promoter region at -2549 site has been identified in the Lahore population. The result of my study was that DD genotype was seen in diabetic patients with retinopathy relative to diabetic patients without retinopathy. The band for DD genotype is analyzed at 211bp and the band for II genotype was seen at 229bp. This 18bp section I/D mutation of vascular endothelial growth factor gene was additionally defined through sequence examination of heterozygous section for I/D mutation. Though, in this study the progressive relationship of the DD gene mutation or D allele was observed as autonomous probability aspect for the development of retinopathy in diabetic people as this research included two groups such as Control (DM) and diseased (DR). Vascular endothelial growth factor (VEGF) is chosen in this investigation because of its significant impacts on retina of eye after the development of diabetic retinopathy. It is seen that extent of vascular endothelial growth factor additionally increments during the cycle of arrangement of fresh blood vessels from remaining new blood vessels in eye in diabetic retinopathy [10]. Hietala *et al.* (2008) reported a hereditary association of proliferative retinopathy in type 1 diabetes patients [11]. This finding indicates the pathology of PDR in type 1 diabetes is a genetic aspect. Approximately all people with type 1 diabetes have been shown to have the extent of retinopathy after living with diabetes for 20 years [12]. The mutation in this gene analyzed in various nations and in Pakistan. Some of analysis are contrasted with distinctive mutation of this gene and their outcomes indicated that vascular endothelial growth factor gene has solid relationship with the advancement of diabetic retinopathy. Various possible polymorphisms have been identified in the VEGF gene. The polymorphism of _2578C/A for molecular testing in the promoter area of VEGF quality was reported, since this mutation was observed in Egyptian people in various groups. VEGF polymorphism has been reported to affect the vulnerability to proliferative diabetic retinopathy. In this study, the results revealed a greater range of the genetic mutantion (C/A and A/A) in both of non-proliferative diabetic retinopathy and the PDR relative to control C/C gene mutation [13]. Buraczynska *et al.* (2007) reported the possible interaction of 18 base pair section Insertion/Deletion mutation of regulatory area at -2549 site in Caucasian population [14]. The specific gene expression shows a central part in the pathogenesis of diabetic retinopathy problems. The higher incidence of DD genetic variation of polymorphism of vascular endothelial growth factor Insertion/Deletion in retinopathy people in Egyptian population relative to the DM people without retinopathy and in control did not display major variations in their genotype expression. They noticed the higher incidence of DD gene mutation in people with DR over 2 ways and greater chance of multiple and half crease with D factor in people with DR relative to normal group [15]. VEGF has three fundamental areas for example promoter area, 3′ untranslated region and 5′ untranslated region. Vascular endothelial growth factor gene is profoundly multifactorial and different mutations are observed on every one of these areas and known as significant gene in the progression of diabetic retinopathy and furthermore induces formation of new blood vessels in different disorders such as joint inflammation, DN and cancer. In this investigation, significant spotlight was on regulatory area. In past reports, six VEGF polymorphisms were detected in a screening report: G(-1877)A, T(-1498)C, G(-1190)A, G(-1154)A in the regulatory area and C(-634)G, C(7)T in the 5′ untranslated region. The other five polymorphisms and C (936) T and G(1612)A mutations in the 3′ untranslated region were identified to be normal in the people of Japan. Past reports have also revealed that the production of VEGF is affected by mutation in both the regulatory region and 3′ untranslated regions of the VEGF [16]. It was reported that the alleles possessing the VEGF -152A (rs13207351) and -116A (rs1570360) were strongly linked with PDR. Three promoters linked with PDR, the single nucleotide polymorphisms 160, 152 and 116. Proliferative stage of diabetic retinopathy is strongly correlated with the _160CC genotype. The genotype _152AA is closely related to PDR (OR_3.5). The _116 SNP displayed the strong correlation in the analysis, with PDR group demonstrating a substantially greater incidence of the AA genotype [17]. The existence of D factor in the regulatory area of vascular endothelial growth factor gene at -2549 position contribute to increased gene expression. Past reports have also revealed that VEGF output is correlated with mutation in the promoter region along with 3′ untranslated region of VEGF gene [18]. The impact of 3 mutations of VEGF on the progression of DME has been investigated in Japanese population. Their findings indicate that polymorphism of VEGF at C -634 G is a contributing hazard predictor for both ME and DR with T2DM [19]. In the regulatory area of VEGF on 405, presence of G factor contributes to increased gene

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expression in Caucasian population. The SNP rs699947 exhibited full association of Insertion/Deletion mutation on -2549 site from interpretation binding facility, that is located 29bp downstream from rs699947 [20]. In alternative investigation performed in north Indian people of Amritsar the Insertion/ Deletion mutation of vascular endothelial growth factor gene has been recognized in irregular bosom malignant growth patients and strikingly they have discovered the critical relationship of II genotype and I allele in bosom disease patients when contrasted with control bunch [21].

CONCLUSION

This study indicates that DD gene mutation and D allele is an autonomous hazard aspect for the advancement and progression of retinopathy in people with type 2 diabetes, additionally different issues for example diabetic age and family ancestry of diabetes assumes key function in the advancement of retinopathy in diabetic patients. In DR subjects 2-11% deletion was observed. Conversely there was huge relationship of II gene mutation or I allele in type 2 diabetic patients.

Conflicts of Interest

The authors declare no conflict of interest

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Original Article

Exploring the Relationship between TNF-α Gene Expression in Non Diabetic Nephropathy Type 2 Diabetes Patients

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ABSTRACT

Non-diabetic nephropathy (NDN) is a common complication of type 2 diabetes, leading to kidney damage and impaired kidney function. TNF-α (tumor necrosis factor-alpha) is a pro-inflammatory cytokine that has been implicated in the development of NDN. **Objective:** To evaluate the gene expression of TNF-α in patients with type 2 diabetes mellitus (T2DM) without nephropathy to gain insight into the potential role of TNF-α in the pathogenesis of diabetic nephropathy (DN). **Methods**: Total of 80 subjects were tested, split into two groups, including healthy patients, T2DM patients without nephropathy, and T2DM patients with nephropathy. RNA was extracted from blood samples, and RT-PCR was used to observe the impact of T2DM without nephropathy on the expression of the TNF-α gene using gene-specific primers and SYBR Green mix. **Results:** The results showed almost 4.4-fold induced expression of TNF-α in T2DM patients without nephropathy compared to the normal group. **Conclusion:** The findings may have implications for the development of new therapies and biomarkers for DN, and for a good interpretation of the complex pathophysiology of T2DM. The exact role of TNF-α in the pathogenesis of DN in humans is not fully understood, and further investigation is needed.

INTRODUCTION

Hyperglycemia brought on by insulin resistance and/or inadequate insulin production characterizes diabetes mellitus, a chronic metabolic condition. With 90% of cases globally, type 2 diabetes mellitus (T2DM) is the most prevalent type of diabetes [1]. T2DM is linked to a number of microvascular and macrovascular consequences, including stroke, retinopathy, neuropathy, and nephropathy [2]. Around 30% of T2DM patients get nephropathy, one of the disease's most prevalent and harmful consequences [3]. Albuminuria, a decreasing glomerular filtration rate (GFR), and structural alterations in the kidney, such as mesangial enlargement, glomerular basement membrane thickening, and interstitial fibrosis, are all signs of the progressive kidney disease known as diabetic nephropathy (DN) [4]. Many variables, including genetic susceptibility, hyperglycemia, dyslipidemia, hypertension, and inflammation, have a role in the aetiology of DN [5]. Inflammation has a significant role in the pathophysiology of DN and other T2DM problems [6]. The pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-alpha) is essential for controlling inflammation and immunity [7]. TNF- is generated by a variety of cells, including monocytes, macrophages, T cells, and adipocytes, and it interacts with a variety of cell types, including endothelial cells, mesangial cells, and podocytes, to cause tissue damage and inflammation [8]. The role played by TNF- in the onset and development of DN has been the subject of several investigations. It has been

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shown that TNF- expression is elevated in the kidney of DN patients as well as in DN animal models [9, 10]. By its impact on endothelial dysfunction, oxidative stress, and fibrosis, TNF- has been linked to the pathophysiology of albuminuria, GFR reduction, and structural abnormalities in the kidney [11, 12]. More research is required since it is unclear exactly how TNF-a contributes to the pathophysiology of DN in people. In order to better understand the possible function of TNF- in the aetiology of DN, we evaluated the gene expression of TNF- in patients with T2DM without nephropathy in this research. Our research may have ramifications for the discovery of novel DN treatments and biomarkers, as well as for a deeper understanding of the intricate pathophysiology of T2DM.

METHODS

It was a cross sectional case-control study. The research work was carried out in the Immunology department and Resource lab UHS, Lahore. The calculated sample size for each group is 40. A total of 80 subjects were tested for this study and they were divided into two groups of 40 individuals in each group. Group-I with 40 healthy patients. Group-II with 40 patients of T2DM patients without diabetic nephropathy. Five ml venous blood was collected in EDTA coated vacationers from T2DM patients with and without nephropathy and was brought to the Resource lab within four hours of the sample collection to avoid genomic RNA degradation. The primers were suspended using low TAE buffer in a calculated amount to achieve concentration 1µg/µl as stock. A working solution of 10pm/µl diluted from stock were used for all further PCR experiments. Primers were optimized for reaction conditions of annealing temperature, Mg concentration, amount of buffer and dNTPs. These optimum conditions were in further experimentation. The following primers was used:

Gene	Primer	GC content (%)	Product Size
TNF α -F	5' CGAGTGACAAGCCTGTAGC 3'	45	45
TNF α -R	5' GGTGTGGGTGAGGAGCACAT 3'	50	3

Table 1: Primer used for PCR

RNA was extracted from blood samples within 6 hours of sample collection. Samples was stored in trizol if extraction is delayed. Samples of extracted RNA were kept at -80°C. RNA quality and quantity were assessed using nanodrop technology. Pcr reaction was followed by gel electrophoresis. The statistical programme SPSS was used for all calculations(version 20.0).

RESULTS

For this research, 80 patients in all were enrolled, and three groups were assigned to them. Group I 40 healthy patients. Group-II 40 patients of T2DM patients without diabetic nephropathy.

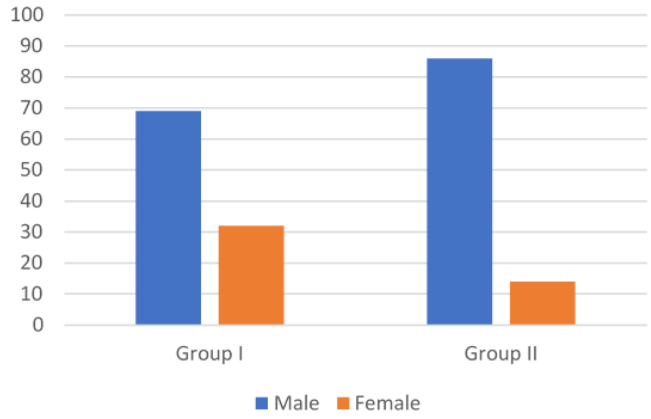

Figure 1: Comparing between Control group and Chronic T2DM

The table 2 presents two groups of 40 individuals each, labeled as Group I and Group II, and provides information about their ages, ALT, and AST levels.

Variables	Group I	Group II
	N= 40	N= 40
Age	47.49 ±2.72	54.99 ± 3.99
ALT	22.71 ± 5.22	66.99 ± 38.47
AST	32.24 ± 7.54	110.09 ± 72.71

Table 2: ALT and AST level in both groups

The figure 2 lists showed subjects along with their corresponding concentration in ng/µl and the amount used for cDNA at 1.5µg.

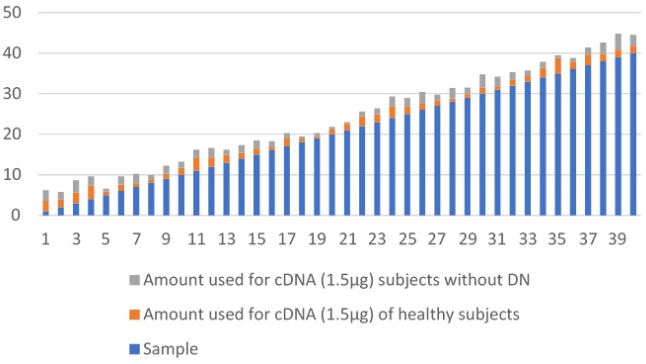

Figure 2: Amount of cDNA used in 2 groups

The impact of T2DM without nephropathy on expression of TNF-a gene was observed by RT-PCR by using gene specific primers and the dye which is SYBR Green mix of the ferments. For internal control GAPDH gene was applied. Each PCR assay of real time was performed in triplicate. Increased expression was observed in T2DM patients with and without nephropathy group as compared to normal group. Almost 4.4 fold induced expression of TNF- a was observed in T2DM patients without nephropathy.

DISCUSSION

TNF-mRNA expression was shown to be low in GMCs that had not yet been triggered by lipopolysaccharide (LPS), according to a research by Affres et al., using the Northern blot technique [13]. In GMC, TNF expression may be induced

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by endotoxin alone as well as by complement fragment, immune complex, platelet-derived growth factor, epidermal growth factor, platelet activating factor, and interleukin [14]. The processes of chronic muscle degeneration and regeneration, which are typical of primary muscle diseases, cause a rise in TNF levels. We aimed to clarify the influence of TNF and IGF1 on gene expression during the early stages of skeletal muscle cell differentiation since pathologically elevated TNF- and IGF1 levels may have either a favourable or detrimental effect on the efficiency of muscle cell differentiation. Many elements, including hormonal imbalances, pathogenic agents, and cytokine gene polymorphisms, control the development and release of cytokines. The genotypes and alleles of the IL-4 gene's -590 region were shown to significantly vary between type 2 diabetes patients with nephropathy and healthy controls [15, 16]. Therefore, based on the current and previous studies, it can be concluded that the expression are associated with nephropathic complications rather than type 2 DM in our studied population by Meloni et al.,[17]. Data showed that there was no relationship between these polymorphisms and type 2 diabetic patients without nephropathy. Many disorders, including glomerulonephritis brought on by immunological complexes, renal lupus, post-aminoglycoside nephritis, rheumatoid arthritis, and septic shock, are mostly caused by TNF. Individual TNF circulating levels differ. Moreover, the rise in circulating TNF levels after the application of an adequate stimulus is substantially larger in some individuals than in others. Activation of the pro-inflammatory cytokine system is elevated in chronic renal failure. Cytokine levels, such as TNF, are significantly elevated in the blood both before and after beginning dialysotherapy. The most significant factor contributing to elevated proinflammatory cytokine levels in dialyzed individuals is likely end-stage renal disease itself [18–20]. It is probably caused by decreased renal clearance and increased cytokine production. Also, it has been shown that TNF- levels are related to the pathological alterations in mesangial proliferative glomerulonephritis (MsPGN), suggesting that TNF- may be a key role in the proliferation, sclerosis, and disease development of GMC. The work by Ozen et al.[16] concentrating on the relationship of TNF- and DN is relevant in light of the significance of TNF- for GMC.

CONCLUSION

In conclusion the implications for the development of new therapies and biomarkers for DN, and for a better understanding of the complex pathophysiology of T2DM.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Article

Influence of different Carbon and Nitrogen Sources on the Production of Single Cell Biomass from Potato Peels

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ABSTRACT

Potato peel can be converted into various value-added compounds, such as enzymes, bio sorbents, biohydrogen, and biogas. **Objective:** To evaluate the influence of different Carbon and Nitrogen Sources on the Production of Single Cell Biomass from potato peels. **Methods:** The process of fermentation was carried out in mix broth with different concentrations of carbon, nitrogen, and different nitrogen sources to determine the effect of these factors on the production of SCP. **Results:** Maximum yield of dry cell biomass (0.251 and 0.245 g/100 ml) was achieved with organic nitrogen source peptone and inorganic nitrogen source ammonium sulphate, respectively. Ammonium sulphate is more suitable to use as peptone is expensive organic nitrogen source. Next optimization of nitrogen concentration was done with ammonium sulphate with different concentrations and best yield (0.190 g/100 ml) was obtained with 1.5% nitrogen concentration. **Conclusions:** In conclusion, the study suggests that ammonium sulphate is a more suitable nitrogen source than peptone for maximizing the yield of dry cell biomass. Additionally, optimization of nitrogen concentration with ammonium sulphate showed that a 1.5% concentration is the best for achieving the highest yield. These findings have important implications for improving the efficiency and cost-effectiveness of industrial-scale production of dry cell biomass.

INTRODUCTION

Using emerging scientific approaches, developing countries can expand their economies by converting their low-cost industrial and agricultural wastes into more valuable products [1]. As potato peel waste increases due to increased consumption of manufactured edible potato products, it can also be converted into various value-added compounds, such as enzymes, bio sorbents, biohydrogen, and biogas [2]. Rapid increase in population is damaging the quality of life and causing shortage of food and resources. Researchers all over the world are putting efforts to control this issue by technological progress. Currently in this global village technology enables us to make precise estimates of how much resources we shall need in future for human livelihood [3, 4]. Two basic factors that will increase demand of food and water in future are: First, increase in population at rate of 1.4, so by 2050 the human population will grow to nearly 9.3 billion people; Second, the development in standard of living which means by 2050 about 3 billion people will join widening middle class, mainly due to economic progress in developing countries [1]. Which will result in changed life style and different food requirements. These changes will cause 50% hike in protein demand and 102% rise in demand of meat products in future. Consequently, tackling such global issues several research studies are focusing new ways of protein production. Making single cell proteins (SCPs) by using agricultural waste source through fermentation is one of the most beneficial approaches [5, 6]. According to literature "SCPs are the dried cells of microorganisms such as fungi, algae and bacteria that are

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used as protein supplement in human foods or animal feeds". By using cheap feedstock and waste products as source of carbon and energy, one can use microorganisms to produce biomass and protein concentrates [7]. Potatoes are produced in large quantity throughout the world. Substantial amount of potato waste is created due to its broad use in different food industries. Potato waste can be used to produce environment friendly industrial goods. Various "green chemistry" techniques can help in extracting polyphenols from potato peels which can have both environmental and economic benefits. Anyhow, further research is necessary to improve processing lines like Investment of capital, use of energy, yield, nature of solvent and integration [8, 9]. Till now no unconventional processes carry out all these requirements and it is still a challenge to produce cost effective products at industrial level [10]. Huge amount of potato crops is produced every year throughout the world and is one of the most important components of human nutrition. Potato peel waste has zero worth as by-product of potato-processing industries. Almost 15-40 % of total potato weight turns into potato peel waste depending upon the different peeling process used by food industries [11]. Such large quantity of food waste creating worries for many scientists especially in Europe and their scientific research provides multiple solutions for the problem. The article focused on summarizing the research work on how potato peel waste can be utilized in food producing industries [12]. Results show that potato peel waste can be used as antioxidant in food chain as it has large phenol content, partial flour substitute and in fermentation as solid substrate [11, 13].

METHODS

The process of fermentation was carried out in mix broth with different concentrations of carbon, nitrogen, and different nitrogen sources to determine the effect of these factors on the production of SCP. To study the effect of different carbon concentrations on the mix broth (100 ml) with different carbon concentration (%) of 1, 2, 3, 4 and 5 were prepared in flasks. These flasks were properly plugged and autoclaved at 121 °C for 15 minutes after adjusting thier pH at 5.5. These flasks were inoculated with *R. oligosporous* under aseptic conditions. These flasks were then incubated at 35 °C for 3 days. After three days, inoculated flasks were filtered with whatmann filter paper and biomass was separated. All the experiments were carried out in triplicates. The influence of diffrent organic (urea, peptone and yeast extract) and inorganic (Ammonium sulphate and Ammonium nitrate) nitrogen sources was tested on the production of single cell biomass by adding each of the nitrogen source into the growth media at 0.5% w/v. The pH of each flask was aadjusted at 5.5 with 1M HCl/ 1M NaOH and then plugged with the cotton. These media were sterlized by autoclaving at 121 °C for 15 minutes. After autoclaving, inoculum prepared from slants of *Rhizopus oligosporous* was added into each flask at 2% v/v in the laminar air flow and incubated at 35°C for three days. After three days, biomass was separated from filtrate by filration by using whatmann filter paper No. 1. All the experiments were carried out in triplicates. In the previous experiments best yield of SCP was achieved with Ammonium sulpahte, therefore in this parameter the effect of different concentarions of Ammonium sulphate was explored. The experiment was carried in triplicate flasks by adding Ammonium sulphate in concentrations (%) of 0, 0.5, 1 and 1.5. The pH of all the media was adjusted with 1M HCL and 1M NaOH at 5.5 and plugged with cotton properly before autoclaving. The media were sterilized by autoclaving at 121°C for 15 minutes and then 1 ml of inoculum was added in each flask under aseptic conditions. All the flasks were incubated at 35 °C and after three days the biomass was collected by the filtration process. The wet biomass was weighed and dried before taking the dry biomass weight. All the experiments were carried out in triplicates.

RESULTS

The effect of different carbon concentrations (0%, 1%, 2%, 3%, 4%, 5%) was evaluated in the fermentation process to get the best quantity of dry cell biomass. The results obtained were described in Table 1. The best yield of single cell protein (0.556 g/100 ml) was acquired with carbon concentration of 3% after three days of fermentation period. The maximum crude protein content of dried biomass obtained was 45-55 %. The stastical analysis showed that the dry biomass with respect to the different concentrations of glucose was highly significant ($p < 0.001$). The clustered bar graph respresnting the total dry biomass, biomass yield and consumed sugar of each concentration of glucose was shown in Figure 1.

Sr. No.	Glucose (%)	Dry biomass (%) Mean ± SD	Consumed sugar (%) Mean ± SD	Biomass yield (g/g) Mean ± SD
1.	1.	$0.33^{d} \pm 0.003$	$1.87^{e} \pm 0.007$	$0.18^{b} \pm 0.003$
2.	2.	$0.40^{c} \pm 0.018$	$2.15^{d} \pm 0.005$	$0.19^{b} \pm 0.005$
3.	3.	$0.56^{a} \pm 0.007$	$2.55^{c} \pm 0.004$	$0.22^{a} \pm 0.004$
4.	4.	$0.45^{b} \pm 0.017$	$4.43^{a} \pm 0.003$	$0.10^{c} \pm 0.013$
5.	5.	$0.45^{b} \pm 0.015$	$4.34^{b} \pm 0.004$	$0.10^{c} \pm 0.006$
	Significance level (95%)	p<0.001		

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Table 1: Effect of different carbon concentrations on the biomass growth

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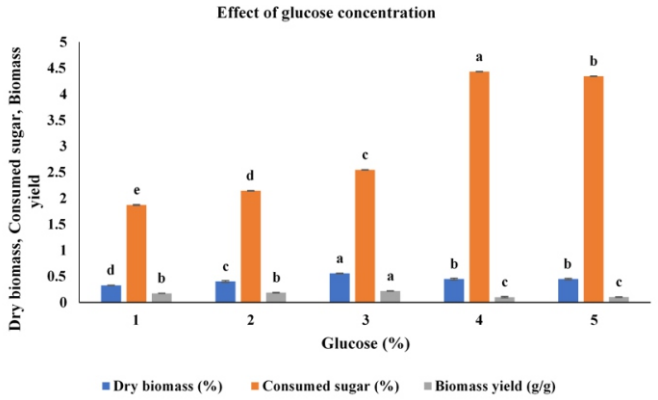

Figure 1: Effect of different glucose concentrations on total biomass yield, total dry biomass and sugar consumed of media

The effect of different organic (urea, peptone, yeast extract) and inorganic (ammonium sulphate, ammonium nitrate) nitrogen sources on the production of single cell protein was studied during the process of submerged fermentation. The results obtained were mentioned in Table 2. which showed that best yield (0.253 g/100 ml) was obtained with ammonium sulphate followed by peptone (0.240 g/100 ml). The total crude protein content of dried cell biomass estimated was in range of 45-55%. The stasistical study showed that dry biomass yield was highy significant with respect to the different nitrogen sources ($p < 0.001$). The clustered bar graph respresnting the total dry biomass, biomass yield and consumed sugar of different nitrogen sources was shown in Figure 2.

Sr. No.	Nitrogen Sources	Dry biomass (%) Mean ± SD	Consumed sugar (%) Mean ± SD	Biomass yield (g/g) Mean ± SD
1.	Ammonium Sulphate	$0.25^{a} \pm 0.007$	$2.59^{a} \pm 0.006$	$0.09^{a} \pm 0.003$
2.	Ammonium Nitrate	$0.23^{b} \pm 0.010$	$2.59^{b} \pm 0.007$	$0.09^{a} \pm 0.007$
3.	Peptone	$0.24^{ab} \pm 0.005$	$2.66^{b} \pm 0.004$	$0.09^{a} \pm 0.004$
4.	Yeast Extract	$0.12^{c} \pm 0.008$	$2.45^{d} \pm 0.003$	$0.05^{b} \pm 0.008$
5.	Urea	$0.06^{d} \pm 0.010$	$2.47^{c} \pm 0.004$	$0.02^{c} \pm 0.005$
	Significance level (95%)	p<0.001		

Means that do not share a letter are significantly different in a column

Table 2: Effect of different nitrogen sources on the biomass yield

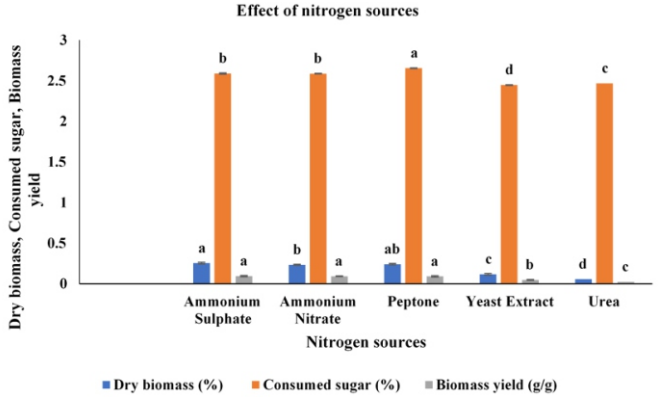

Figure 2: Effect of different nitrogen sources on the yield of dry cell biomass and total sugar consumed

The influence of different nitrogen concentrations (0%, 0.5%, 1%, 1.5%) were studied to oobtain maxium yield. The results obtained were mentioned in Table 3. the results showed that maxium yield of biomass (0.190 g/100 ml) was obtained with nitrogen contentaion of 1.5 %. The crude protein content of dried cell biomass was in range of 45-55%. The statistical study showed that the effect of different nitrogen concentrations on the dry biomass yield was highly significant ($p < 0.001$). The clustered bar graph respresnting the total dry biomass, biomass yield and consumed sugar of different concentrations of nitrogen (ammonium sulphate) was shown in Figure 3.

Sr. No.	Nitrogen Concentration (NH4)2SO4 (%)	Dry biomass (%) Mean ± SD	Consumed sugar (%) Mean ± SD	Biomass yield (g/g) Mean ± SD
1.	0	$0.01^{c} \pm 0.006$	$2.27^{d} \pm 0.005$	$0.00^{a} \pm 0.004$
2.	0.5	$0.16^{b} \pm 0.004$	$2.66^{c} \pm 0.005$	$0.06^{a} \pm 0.008$
3.	1	$0.15^{b} \pm 0.014$	$2.69^{b} \pm 0.004$	$0.06^{a} \pm 0.005$
4.	1.5	$0.19^{a} \pm 0.007$	$2.74^{a} \pm 0.009$	$0.07^{a} \pm 0.006$
	Significance level (95%)	p < 0.001		

Means that do not share a letter are significantly different in a column

Table 3: Effect of different nitrogen concentrations on the SCP yield

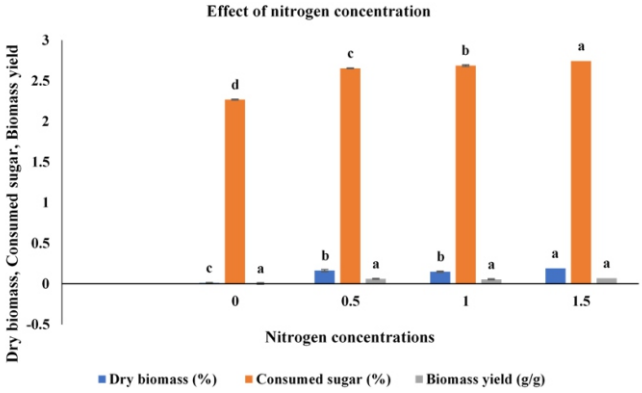

Figure 3: Effect of different concentration of Ammoinium sulphate on the production of dry cell biomass and total sugar consumed

DISCUSSION

The factors that influence the production of dry cell biomass are mainly the type of substrate, the type of microorganisms, inoculum age and size, composition of media, incubation temperature, pH, incubation time, nitrogen sources, carbon concentration, heat removal, aeration as well as agitation speed *etc.*, [14, 7]. Different studies showed that particle size and quantity of nitrogen source are the factors that greatly influence biomass production and its protein content [15]. In our study different nitrogen sources were tested to achieve better quantity of dry cell biomass and ammonium sulphate gave the best yield at 1.5 % w/v. In case of inorganic nitrogen source ammonium sulphate gave the highest yield while in

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case of organic nitrogen source peptone provided with best growth. Similar case happened when ammonium sulphate was added into the fermentation media then maximum yield of biomass was obtained with *A. niger* and *S. cerevisiae*. Ammonium sulphate not only provide nitrogen but also Sulphur to the medium for better growth [16]. Yousufi (2012), attained the maximum content of protein (61.2 mg/100 g) on fruit waste through solid state fermentation from *Rhizopus oligosporus* while in our study 45-55% crude protein was achieved from dry cell biomass of *R. oligosporus* [17]. The increase in our protein content may be due to supplementation with nitrogen source. Nguyen *et al.*, obtained maximum protein content of (39.71%) *Rhizopus oligosporus* biomass by using the cassava as a substrate with different quantities of macro and micro nutrients under variable conditions [18]. In our study 50% crude protein content was obtained with same fungus but different substrate with diverse compositions and under different conditions. The difference may be due to change in substrate, micro, macro supplements and conditions [19, 20].

CONCLUSIONS

Different factors affecting production of biomass from potato peels was studied in this research. Since potato peels are a good source of sugar and other nutrients required for microorganisms to survive, potato peels can be considered an attractive substrate for the production of single cell protein. In addition to adding nitrogen to basic media, it is also possible to boost the yield of dry cell biomass by supplementing it with nitrogen. Additionally, animals can be fed SCP produced from other cheaper agro-industrial sources. As a result of reducing the cost of protein-rich meals used as feed for animals through the use of cheaper and waste resources, the environmental pollution issue will be minimized. In comparison to proteins obtained from agriculture, single cell proteins are a better alternative.

Conflicts of Interest

The author declare no conflict of interest.

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Original Article

Assessment of Risk Factors of Septicemia

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ABSTRACT

Septicemia, commonly referred to as blood poisoning, is a potentially life-threatening medical condition caused by the presence of harmful bacteria in the bloodstream. **Objective:** To assess risk factors of septicemia and observe correlation between them. **Methods:** This study is cross-sectional, descriptive, and observational. From the medical wards and Accident & Emergency Department of Mayo Hospital Lahore, 101 patients with septicemia were removed. Data was entered into a Performa created specifically for this use. **Results:** 15/32 patients of UTI, 11/18 patients of bed sores, 6/13 patients having chest infection, 4/8 patients having wound infection, 4/6 patients of hepatic encephalopathy and 3/6 patients of CVA were diabetic. Correlation at two levels was seen i.e., 0.05 which is an indicative of significant correlation and 0.01 which indicates highly significant correlation. **Conclusions:** Understanding the risk factors associated with septicemia is crucial for preventing and managing this condition. Age, male sex, diabetes, smoking, hypertension, and immunocompromised status have all been identified as significant risk factors for septicemia. Improving early life conditions, increasing vitamin D intake, and improving nutritional status may also play a role in reducing the risk of septicemia.

INTRODUCTION

Septicemia, commonly referred to as blood poisoning, is a potentially life-threatening medical condition caused by the presence of harmful bacteria in the bloodstream [1]. It is a major health risk that can result in sepsis, a disease when the immune system of the body overreacts to an infection, producing widespread inflammation and possibly even organ failure [2, 3]. People of all ages can develop septicemia, which can be brought on by a variety of different bacterial infections. The etiological factors of septicemia include alcohol [4], old age, socioeconomic status, lowered immunity, immunocompromising diseases (including AIDS and other hematological malignancies) [5, 6], diabetes mellitus (DM) [7, 8], HIV, hemodialysis [9], hospital acquired infections [10], cirrhosis [11], and comorbidity with several chronic and infectious diseases [12]. One of the most prevalent chronic co-morbid medical diseases in the USA is diabetes mellitus (DM), which is usually present in sepsis patients [7, 8]. Granulocytopenia, which commonly coexists with severe bacterial infection in alcoholics and is negatively correlated with clinical prognosis. Alcohol reduces granulocyte production during septicemia and inhibits the stem cell antigen-1 response in granulocyte lineage-committed precursors, which may be an unique mechanism causing decreased host defense in alcoholics [4]. Patients receiving both hemodialysis (HD) and peritoneal dialysis (PD) commonly develop septicemia [13]. Age-related mortality from septicemia rises dramatically. Improved prenatal environments may facilitate better adaptive immunity development, hence boosting immunity against bacterial infections [6].

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Systemic infections are rare and only occur in elderly people or those with severe underlying diseases, such as cirrhosis [11]. Assessing the risk factors for septicemia is critical in preventing and treating this condition. Understanding the factors that increase the likelihood of developing septicemia can help healthcare providers identify patients who are at risk and take appropriate measures to prevent or manage the condition [14, 15]. This article explored the various risk factors for septicemia, including underlying medical conditions, invasive medical procedures, and weakened immune systems. By examining these risk factors, we can gain a better understanding of how to prevent and manage septicemia, ultimately improving patient outcomes and reducing the incidence of this serious condition.

METHODS

This study is cross-sectional, descriptive, and observational. From the medical wards and Accident & Emergency Department of Mayo Hospital Lahore, 101 patients with septicemia were removed. Children, pregnant women, and women nursing infants were not allowed to participate in the study. Individuals above the age of 30 were enrolled. These individuals have had in-depth examinations to identify the origin and etiology of the disease at the time of presentation. The patients' or their guardians' permission was obtained. Throughout the data gathering procedure, every other ethical concern was taken into account. Also, the KEMU Ethical Consideration board gave their approval. Data was entered into a Performa created specifically for this use. liver function tests, renal function tests, and full blood counts were performed for each patient. SPSS latest version was used statistical analysis of data.

RESULTS

15/32 patients of UTI, 11/18 patients of bed sores, 6/13 patients having chest infection, 4/8 patients having wound infection, 4/6 patients of hepatic encephalopathy and 3/6 patients of CVA were diabetic (Table 1).

Causes	Diabetics	Non-diabetic	Total
Urinary Tract Infection	15	17	32
Bed Sores	11	07	18
Chest Infection	06	07	13
Wound Infection	04	04	08
Diabetic Foot	04	00	04
Brain Injury	00	05	05
Hepatic Encephalopathy	04	02	06
Gastroenteritis	03	01	04
Aspiration Pneumonia	01	05	06
Old CVA	03	03	06

Table 1: Major causes of Septicemia with respect to Diabetes status

Correlation was seen between different variables (Table 2). Pearson's correlation was applied which shows correlation at two levels i.e., 0.05 which is an indicative of significant correlation and 0.01 which indicates highly significant correlation. Negative sign (-) before a numeric value shows that two variables are inversely/negatively correlated. ALP and HTN are negatively whereas Glucose is positively correlated with age of patients.

Correlating Variables		Pearson's Correlation at 0.05 level	Pearson's Correlation at 0.01 level	Sig. (2tailed)
Age of Patients	ALP	-.215		.031
	B. Glucose	.248		.012
	Hypertension		-.295	.003
Smoking	B. Urea	.224		.024
Upper GI Bleeding	Bilirubin		-.269	.007
Septic Shock	B. Urea		-.263	.008
Chest Infection	Albumin	.203		.042
Urinary Tract Infection	WBC	.217		.030
Platelets Count	B. Glucose	-.205		.040
Chronic Renal Failure	Na+		.334	.001
Chronic Renal Failure	K+		-.290	.003
Respiratory Failure	ALT		-.759	.000
Respiratory Failure	AST		-.684	.000
End Stage Renal Disease	B. Urea		-.310	.002

Table 2: Correlation between Biochemical and Hematological parameters, Risk factors, Complications and Causes of Septicemia

Table 3 shows major risk factors of septicemia observed in this study. Diabetes was observed in 45 participants in which 24 were male and 21 were female. Hypertension was observed in 23 participants in which 13 were male and 10 were female. Smoking was observed in 18 participants all of which were males.

Major Risk Factors	Male	Female	Total
Diabetes	24	21	21
Hypertension	13	10	10
Smoking	18	00	00

Table 3: Major Risk Factors of Septicemia

DISCUSSION

Age, male sex, a history of diabetes, smoking one pack or more cigarettes per day, and difficulty performing daily tasks are risk factors that significantly and independently raise the fatality rate in septicemia [16]. Diabetes makes a person more vulnerable to septicemia and infection [17]. One of the most prevalent etiological variables is diabetes, which is usually present in sepsis patients [7]. The most prevalent risk factor in our analysis was diabetes, which was present in 44.55% of patients. Diabetics made up 42% of women and 47.05 % of men. Smoking has damaging effects on the skin, soft tissues, respiratory system, and immunological system, and it raises the risk of septicemia

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[18]. After smoking, hypertension was the second often observed risk factor in our study. Men and women experienced somewhat different rates of septicemia [8]. In our study, septicemia afflicted males and females equally (50.50% males, 49.0% females). The tests performed for this study exhibited identical findings for both genders, with the exception of ALT, which was normal in the majority of males (64.70%) but increased in the females (62%). Patients with weakened immune systems are more likely to acquire bloodstream infections such septicemia [19]. The development of adaptive immunity is improved by better early life settings, which may increase immunity (resistance) against bacterial infections [6]. Vitamin D and sun UVB are essential factors in lowering the incidence of septicemia, according to a US based research [12]. The risk of septicemia can be reduced by improving nutritional status [20]. A patient should also maintain good personal hygiene. Cuts, pricks, and surgical wounds need to be managed properly. In addition to preventative antibiotics and routine checkups, proper medical care is also required.

CONCLUSIONS

In conclusion, septicemia is a serious and potentially life-threatening condition that can affect people of all ages. Understanding the risk factors associated with septicemia is crucial for preventing and managing this condition. Age, male sex, diabetes, smoking, hypertension, and immunocompromised status have all been identified as significant risk factors for septicemia. Improving early life conditions, increasing vitamin D intake, and improving nutritional status may also play a role in reducing the risk of septicemia. It is important for patients to take care of their personal hygiene and for healthcare providers to manage surgical wounds and cuts properly. Regular medical checkups and prophylactic antibiotics can also help prevent septicemia. By identifying and addressing these risk factors, we can work towards reducing the incidence of septicemia and improving patient outcomes.

Conflicts of Interest

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Original Article

Computational Prediction of *Cymbopogon Citratus* Compounds as Promising Inhibitors of Main Protease of SARS-CoV-2

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ABSTRACT

There is a dire need to develop any antiviral therapy for the treatment of SARS-CoV-2. **Objective:** To investigate the potential therapeutic drug agents from *Cymbopogon citratus* compounds against the main-protease (M^{pro}) of SARS-CoV-2. **Methods**: Initial screening was carried out using molecular docking, dynamic simulation followed by ADMET profiling and Lipinski's physiochemical parameters for prediction of drug likeliness. MOE/PyRx was used for docking before determining the stability of the best complexes through NAMD/VMD softwares. Moreover, SwissADME and admetSAR web-based tools were used for drug likeliness of the best complexes. **Results:** Out of total 50 compounds, 11 presented the lowest binding energies which includes tannic acid, isoorientin, swertiajaponin, chlorogenic acid, cymbopogonol, warfarin, citral diethyl acetal, citral acetate, luteolin, kaempferol and cianidanol with binding energies of -8.12, -7.38, -7.33, -6.88, -6.48, -6.32, -6.31, -6.18, -6.18, -6.13 and -6.02, respectively. Current studies show isoorientin, chlorogenic acid and tannic acid as the promising drug agents using RMSD, Hbond, heatmap graphs. **Conclusion:** Further in-vivo experiments are suggested to ascertain the medicinal use of these potential inhibitors against COVID-19.

INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, is an enveloped positive-sense, single-stranded RNA virus that has the potential to cause infections in the respiratory, gastrointestinal, nervous, and hepatic systems in humans [1, 2]. The novel coronavirus emerged in Wuhan, China, in late 2019 during an outbreak and it belongs to the same family as Middle East Respiratory Syndrome Coronavirus (MERS) and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) [3]. The novel coronavirus transmit human-to-human through aerosol and uses Angiotensin-converting enzyme 2 (ACE2) receptor to cause infectivity [4]. The symptoms of COVID-19 infection appear after approximately 5.2 days and include cough, fever, fatigue or myalgia, pneumonia, and dyspnea [2]. Currently, there is a lack of any antiviral therapy for the treatment of COVID-19. However, the limited measures that have been implemented include many supportive and preventive therapies to prevent organ damage and further complications [5]. The main protease, also known as

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3CLPro, is a key enzyme of SARS-CoV-2 that plays a major role in viral replication and transcription [6]. The Mpro processes the ORF1ab polyprotein by recognizing a specific motif sequence and producing smaller functional proteins that are essential for viral replication [7]. The M^{pro} is a potential target for designing antiviral drugs that can inhibit the enzyme's activity and prevent viral replication [8]. The $3CL^{Pro}$ is composed of anti-parallel, six-stranded β-barrels at the substrate-binding site that span through the protease-like picornavirus 3C domain II and protease-like chymotrypsin-3C domain I [9]. The dimerization process is regulated by the globular structure of five helices that are present between residues 198-303 in domain III, and this process occurs through salt bridge interactions between Arg4 and Glu290 [6, 10]. Many compounds derived from medicinal plants have been reported to have antiviral activity. One such plant is Cymbopogon citratus, commonly known as lemongrass, which belongs to the Gramineae family [11, 12]. Lemongrass possesses a range of pharmacological activities, including antimicrobial, antifungal, anti-inflammatory, antioxidant, anti-nociceptive, anti-diarrheal, and anti-obesity properties [13-15]. These properties are due to the presence of essential oils, flavonoids, phenolic compounds, and other phytochemical constituents in the herb [16, 17]. In this study, the potential inhibitors from *C. citratus* compounds were predicted against SARS-CoV-2 main-protease using molecular docking and dynamic simulation analysis. Main-protease was docked and redocked with C. citratus compounds using Molecular Docking Environment (MOE) and PyRx software to validate results. The binding energies of different compounds were calculated and potential inhibitors were scrutinized for further analysis. The stability and conformational changes of the complexes were calculated by molecular dynamic simulations using NAMD/VMD software. Additionally, the physicochemical and drug likeliness properties of the ligands was calculated using SwissADME, admetSAR and Pfizer's rule of five.

METHODS

Dataset preparation

A library of 50 compounds from *Cymbopogon citratus* was prepared having antiviral, antimicrobial, anti-fungal, anti-inflammatory, antioxidant, and anti-nociceptive properties. 3D-structures were retrieved from PubChem, DrugBank databases or by sketching on chemdraw. Prior to any analyses some preparatory changes were made in ligands structure like addition of hydrogen, assigning charges and energy minimization by universal force field (UFF) with conjugate gradient algorithm of 500 iteration. The chemical compounds of *Cymbopogon citratus* are given in Supplementary Table S1. The main protease also abbreviated as $3CL^{Pro}$, is the key enzyme in SARS-CoV-2 that has a main role in viral replication and transcription. The 3D-crystal structure of targeted protein was retrieved from Protein Data Bank (PDB ID: 6LU7). Beside the removal of repeated chains, heteroatoms, water molecules and already attached ligand, the missing hydrogen atoms were added to make correct protein conformer. Energy minimization was done with Chimera using AMBER forcefield (AMBER ff14SB). The properties of main-protease are given in Supplementary Table S2 while crystal structure is shown in Supplementary Figure S1.

Virtual library screening of Cymbopogon citratus against SARS-CoV-2 M^{pro}

Virtual screening of selected dataset was performed by MOE and PyRx to validate results. The amino acid sequence of active site of protein was either retrieved from literature or using automatic active site finder. Grid box was created with dimension of 20×20×20 Å around the active site of protein and ligand to specify docking. PyRx uses Auto-dock vina to perform docking and computing binding energy by calculating difference between the sum of energy in free state of ligand and protein and sum of energy in protein-ligand complex using AMBER3. The binding energy (ΔG) of protein-ligand interactions was calculated using following empirical equation [18].

$$\Delta G = (V^{L\text{-}L}{}_{bound} - V^{L\text{-}L}{}_{unbound}) + (V^{P\text{-}P}{}_{bound} - V^{P\text{-}P}{}_{unbound}) + (V^{P\text{-}L}{}_{bound} - V^{P\text{-}L}{}_{unbound} + \Delta S_{conf})$$

where *P* refers to the protein, *L* refers to the ligand, $V^{L\text{-}L}{}_{bound}$ energy in bounded state of ligand, $V^{L\text{-}L}{}_{unbound}$ energy in unbounded state of ligand, $V^{P\text{-}P}{}_{bound}$ energy in bounded state of protein, $V^{P\text{-}P}{}_{unbound}$ energy in unbounded state of protein, $V^{P\text{-}L}{}_{bound}$ energy in bounded state of protein and ligand, $V^{P\text{-}L}{}_{unbound}$ energy in unbounded state of protein and ligand, ΔS_{conf} denotes the loss of conformational entropy upon binding.

Calculation of properties using ADMET analysis

The pharmacokinetic and pharmacological properties of ligands which gave best binding scores were calculated to understand the pharmacokinetic role. AdmetSAR and pkCSM web-based tools were used for this purpose.

Molecular dynamic simulations of the top-scoring ligand-protein complex

The molecular dynamic (MD) simulation was performed using NAMD software to examine complex stability and flexibility by allowing to interact in virtual environment similar to human body. Ligands that gave the best docking scores against target protein and have pharmacological properties were selected for post docking analysis. The protein-ligand complex was prepared in same orientation with maximum score for simulation studies. The coordinates of best ligand having highest binding score was embedded in the protein file. The VMD software was

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used to build the topologies of ligand and protein to define bonds and angles, number of molecules and atom types in simulation system. The simulation inputs of ligands were built from CHARMM-GUI web server with CHARMM36 forcefield. The VMD software consists of Automatic PSF Generation which was utilized to convert the protein structure to PSF format. Afterwards, both files were merged and solvated to create a cubic box of water around the complex. Molecular dynamic simulation was run at 0.1 ns (50,000 steps). The energy minimization was done using conjugate gradient method. The periodic boundary conditions were established for energy minimized complex to run simulation. A constant temperature 310K and 1 atm pressure was established for the simulation process. After adjusting all the parameters, the MD simulation was executed using NAMD software. Afterwards, the results were analyzed by plotting histogram of RMSD, hydrogen bond and heat map.

RESULTS

Molecular docking using MOE

Molecular docking is a computational approach used to study the interactions between protein and ligands. In this study, the main protease was docked with 50 compounds of *Cymbopogon citratus* to predict the potential drug agents. The binding energies are listed in Supplementary Table S3. The negative values of binding energy define the release of energy during docking, considering the ligands having high affinity for target protein. The 2 and 3-dimensional interaction of *C. citratus* compound which gave the highest score with main protease is given in Figure 1. while rest of ligands interaction are given in Supplementary Figure S2.

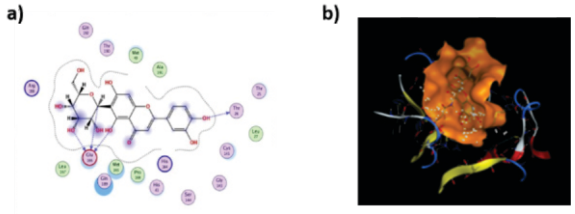

Figure 1: Two dimensional and three-dimensional interactions of Isoorientin with 6LU7

Redocking using PyRx

The three top ligands based on the lowest binding scores with M^{pro} using MOE were selected for further analysis. Before MD simulation, these ligands were first redocked with M^{pro} using PyRx to validate the results since both softwares uses different algorithms. The binding scores obtained from docking are listed in Table 1.

Compounds	Docking Scores from MOE	Docking Scores from PyRx
Tannic acid	-8.12501907	-6.6
Chlorogenic acid	-6.88325262	-6.8
Swertiajaponin	-7.33355188	-5.8
Isoorientin	-7.38438511	-6.7
Warfarin	-6.32509041	-7.5
Citral diethyl acetal	-6.30906057	-4.6
Citral acetate	-6.18121672	-5.0
Luteolin	-6.17798948	-7.1
Kaempferol	-6.13175917	-6.7
Cianidanol	-6.02349663	-6.8

Table 1: The binding energies (ΔG) of 10 compounds of C. citratus with the main protease

Drug likeliness and toxicity risk prediction

The drug likeliness attributes, pharmacokinetics and pharmacological properties of 11 compounds that gave the lowest binding scores were computed using Lipinski's rule and ADMET profiling.

Lipinski's rule of five

The Lipinski's rule physiochemical parameters were studied using the admetSAR and results are shown in Table 2. Out of 11 compounds, only tannic acid showed three violations having molecular weight, number of hydrogen bond donors, and number of hydrogen bond acceptors greater than 500Da, 5 and 10 respectively while rest of the ligands including isoorientin, swertiajaponin, chlorogenic acid and cymbopogonol showed two or less violations.

Ligand	Molecular Weight (<500Da)	logP (<5	H-Bond Donor (5)	H-Bond Acceptor (<10)	Violations
Tannic acid	636.47	-0.28	11	18	3
Isoorientin	448.38	-0.20	8	11	2
Swertiajaponin	462.40	0.10	7	11	2
Chlorogenic acid	354.31	-0.65	6	9	1
Cymbopogonol	426.72	8.02	1	1	1
Warfarin	308.33	3.61	1	4	0
Citral diethyl acetal	226.36	4.08	0	2	0
Citral acetate	210.27	2.77	0	3	0
Luteolin	286.24	2.28	4	6	0
Kaempferol	286.24	2.28	4	6	0
Cianidanol	290.27	1.55	5	6	0

Table 2: The Lipinski's physiochemical parameters of the selected compounds of C. citratus calculated by admetSAR profiling

ADMET profiling

The pharmacokinetic and pharmacological properties of 11 compounds were investigated through ADMET profiling by pkCSM as given in Table 3.

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Compounds	Abs.	Distribution		Metabolism							Excretion		Toxicity	
	IA (%)	VDs log (L/kg)	BBBP (Log BB)	CYP Inhibitor (Y/N)					CYP Substrate (Y/N)		Total Clearance log (ml/min/kg)	ROS (Y/N)	AMES Toxicity (Y/N)	LD50 (mol/kg)
				1A2	2C19	2C9	2D6	3A4	2D6	3A4				
Tannic acid	0.52	0.698	-3.56	N	N	N	N	N	N	N	0.417	Y	N	2.483
Isoorientin	61.76	1.603	-1.56	N	N	N	N	N	N	N	0.372	N	N	2.55
Swertiajaponin	43.48	1.204	-1.82	N	N	N	N	N	N	N	0.421	N	N	2.59
Chlorogenic acid	36.37	0.581	-1.40	N	N	N	N	N	N	N	0.307	N	N	1.973
Cymbopogonol	95.93	-0.14	0.75	N	N	N	N	N	N	Y	0.172	N	N	2.636
Warfarin	96.16	-0.266	-0.17	Y	Y	Y	N	Y	N	Y	0.719	N	N	1.773
Citral diethyl acetal	95.16	0.213	0.668	N	N	N	N	N	N	N	1.787	N	N	1.864
Citral acetate	95.24	-0.064	0.559	N	N	N	N	N	N	N	1.035	N	N	1.812
Luteolin	81.1	1.153	-0.90	Y	N	Y	N	N	N	N	0.495	N	N	2.455
Kaempferol	74.2	1.274	-0.93	Y	N	N	N	N	N	N	0.477	N	N	2.449
Cianidanol	68.82	1.027	-1.05	N	N	N	N	N	N	N	0.183	N	N	2.428

Table 3: The ADMET properties of selected compounds of C. citratus predicted by pkCSM (IA: Intestinal Absorption, VDss: Volume of Distribution in humans, BBBP: Blood-Brain Barrier Permeability, ROS: Renal Organic Cation Transporter 2 Substrate), Abs: Absorption

Furthermore, the bioavailability scores, synthetic accessibility, and other physiochemical properties of virtually screened *C. citratus* compounds were also predicted using SwissADME online tool and are listed in Table 4.

Ligand	TPSA (Å^2)	Bio-availability Score	Synthetic accessibility	Log S	Rotatable Bonds
Tannic acid	310.66	0.17	5.32	-1.624	7
Isoorientin	201.28	0.17	5.04	-2.398	3
Swertiajaponin	190.28	0.17	5.12	-2.302	4
Chlorogenic acid	164.75	0.11	4.16	-2.457	5
Cymbopogonol	20.23	0.55	5.52	-4.414	1
Warfarin	67.51	0.55	3.79	-3.953	4
Citral diethyl acetal	18.46	0.55	3.43	-3.155	8
Citral acetate	43.37	0.55	2.84	-2.918	6
Luteolin	111.13	0.55	3.02	-2.999	1
Kaempferol	111.13	0.55	3.14	-3.142	1
Cianidanol	110.38	0.55	3.50	-3.101	1

Table 4: The bioavailability scores, synthetic accessibility and physiochemical properties of virtually screened C. citratus compounds predicted by SwissADME

Molecular dynamics simulation analysis

The three compounds with highest score were selected for MD simulation due to its lowest binding energy score. The stability and flexibility of newly formed complex was examined by accessing the fluctuations in root mean square deviation (RMSD) values.

RMSD analysis

Visual Molecular Dynamics (VMD) software was used to find out the average distance between atom groups by calculating RMSD values. The RMSD graph of tannic acid in complex with 6LU7 shows quite stability from 450-850 frames with less than 0.2 Å fluctuation. The complex remained stable most of the time at 1.4 to 1.6 Å between 200-900 frames and showed comparatively greater fluctuation at the start and end of simulation while the graphs of isoorientin and chlorogenic acid complexes showed greater stability for longer intervals. The RMSD values of isoorientin complex fluctuated between 1.3Å to 1.6 Å from 25-1000 frames. An abrupt change in values can be observed in the first half between 50-500 frames, however, the second half of simulation from 500-1000 frames showed more stability with less fluctuation. The chlorogenic acid complex showed promising stability between 1.6 Å and 2 Å with less than 0.5 Å deviation from 50-1000 frames. The RMSD graphs of isoorientin, chlorogenic and tannic acids are shown in Figure 2.

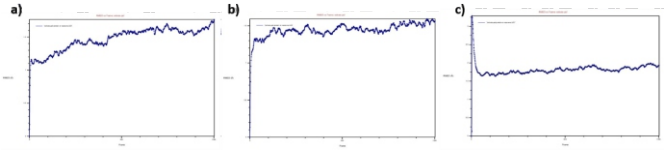

Figure 2: The RMSD graph of **a)** tannic acid **b)** isoorientin **c)** chlorogenic acid in complex with the main protease (PDB ID: 6LU7)

Analysis of hydrogen bonds

The stability of complexes was investigated by plotting histogram of hydrogen bond. The H-bond graphs of tannic acid, isoorientin and chlorogenic acid are shown in Figure 3. The formation of strong hydrogen bond reduces the gap between residues and therefore increases the stability of complex. The hydrogen bond analysis showed that tannic acid complex has total 7 hydrogen bonds, and the stability of complex is provided by the bond between ligand (donor) and Gln127 (acceptor) with 52.24% occupancy rate. Out of 7 hydrogen bonds in isoorientin complex, the bond between ligand (donor) and Glu290 (acceptor) is responsible for stability of complex which remained steady for 62.41% time of simulation process. The chlorogenic acid complex had total 10 hydrogen bonds with maximum stability due to the bond between ligand (donor) and Asp153 (acceptor with 152.94% occupancy.

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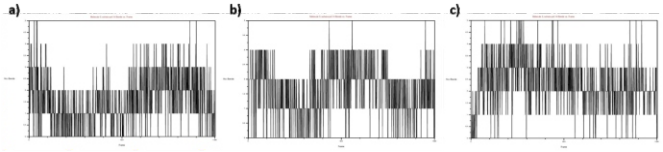

Figure 3: H-bond graph of a) tannic acid b) isoorientin c) chlorogenic acid in complex with the main protease (PDB ID: 6LU7)

Complex analysis using heat maps

The heat map plots represent the distribution of a particular property, such as the potential energy, temperature, pressure and the density of simulated system over time. The heat signatures of isoorientin and chlorogenic acid showed quite stability whereas the tannic acid depicted low stability within short intervals. The heat map graph of isoorientin, chlorogenic and tannic acids are shown in Figure 4.

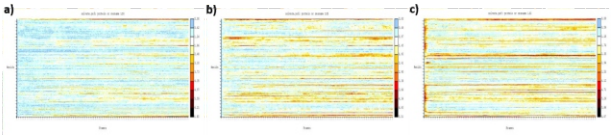

Figure 4: Heat map of **a)** tannic acid **b)** isoorientin **c)** chlorogenic acid in complex with the main protease (PDB ID: 6LU7)

DISCUSSION

The unavailability of antiviral therapy for the treatment of novel SARS-CoV-2 to date is alarming. One of the major limitations in drug discovery is due to RNA nature of its genome which mutates at a faster rate reducing the efficacy of drugs and vaccines. The present study aimed to investigate the potential therapeutic drug agents using molecular docking and dynamic simulation approaches from *Cymbopogon citratus* bioactive compounds against the main protease which is a potential drug target of SARS-CoV-2. The structures of total 50 *Cymbopogon citratus* compounds were identified and retrieved from literature and databases respectively. Their binding energies with the target protein were calculated through docking/redocking using MOE/PyRx. The stability and drug likeliness of ligands which presented lowest binding energies with viral protein was further evaluated through MDS and ADMET analysis. The *In-silico* analysis predicted isoorientin and chlorogenic acid as the potential inhibitor. Similar studies have been reported in the past in predicting the potential inhibitor of this virus. Recent study predicted the promising inhibitor against main protease from the compounds of *Olea europaea* and *Curcuma longa* [19]. Current study was limited at the computational analysis as for MDS, systems with high computation powers are required while our entire analysis was done using personal computer [20]. Analysis using system with high computation power are required to reveal the precise stability of these complexes for higher time frames.

CONCLUSIONS

In-silico drug prediction identified several potential inhibitors of SARS-CoV-2 main protease, including isoorientin, swertiajaponin, cymbopogonol, and luteolin. However, further in-vivo investigations studies are necessary to confirm the medicinal use of these potential inhibitors.

Conflicts of Interest

The authors declare no conflict of interest.

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