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Microbiology and Pathology



Highlights

- Study of α-Amylase Inhibitory Tertiary Referral Hospital in Riyadh
- Fine Needle Aspiration Cytology Detection of Malignancy in Thyroid Cells

Discovering Thoughts, Inventing Future

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Automated Cytopathology of Fine Needle Aspiration for the Detection of Malignancy in Thyroid Cells

By Gabrielly Pereira Ribeiro, Carlos Musso & Dominik Lenz

Pharmaceutical Sciences University

Abstract- Cytopathology of thyroid cells is an established method to detect malignancies in the thyroid. It is of advantage because an anesthesia and a diagnostic laparotomy is not necessary. There are however not yet many studies about automated cytopathology in thyroid cells. To this end, the aim of the present study was to establish an automated diagnosis of malignancy using image analysis and subsequent machine learning and Artificial intelligence. Light microscopy images of 52 patients were analyzed and the results were compared to those of pathology. The results of the automated analysis yielded a sensitivity of 0.94 and a specificity of 0.91 when compared to those of the pathologic diagnoses. The process of machine learning yielded an under curve area of 0.91 as calculated by a ROC-curve. The software used for image analysis, machine learning and classification (diagnosis) are open-source software, respectively.

Keywords: machine learning; cellprofiler; image cytometry; cytopathology; papillary thyroid carcinoma.

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Automated Cytopathology of Fine Needle Aspiration for the Detection of Malignancy in Thyroid Cells

Gabrielly Pereira Ribeiro [α], Carlos Musso [σ] & Dominik Lenz [ρ]

Abstract- Cytopathology of thyroid cells is an established method to detect malignancies in the thyroid. It is of advantage because an anesthesia and a diagnostic laparotomy is not necessary. There are however not yet many studies about automated cytopathology in thyroid cells. To this end, the aim of the present study was to establish an automated diagnosis of malignancy using image analysis and subsequent machine learning and Artificial intelligence. Light microscopy images of 52 patients were analyzed and the results were compared to those of pathology. The results of the automated analysis yielded a sensitivity of 0.94 and a specificity of 0.91 when compared to those of the pathologic diagnoses. The process of machine learning yielded an under curve area of 0.91 as calculated by a ROC-curve. The software used for image analysis, machine learning and classification (diagnosis) are open-source software, respectively. It may be concluded that the method presented in the present study may be used for a low-cost verification of pathological diagnosis. The equipment used for this study (light microscopy, digital camera and a Personal Computer) is available in almost every laboratory.

Keywords: machine learning; cellprofiler; image cytometry; cytopathology; papillary thyroid carcinoma.

This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Brazil.

I. Introduction

Histopathology (HP), a technique that aims to diagnose diseases in tissue sections using microscopy, established in 1893, remains the gold standard until nowadays (Tiford et al., 2006; den Baaker, 2017). In 1928, Papanicolau described a new technique of cell-based diagnosis called Cytopathology (CP) (Van den Tweel and Taylor, 2010).

Results, advantages and disadvantages of HP and CP have always been subject of comparison (Crino et al., 2021; Gerstner, Tarnok, 2002; de Ruiter et al., 1994). Recent literature reveals that the results of HP and CP in terms of accuracy became comparable, independent if CP is conducted manually (de Ruiter et al., 1994) or automatically (Gerstner et al., 2002; Gerstner et al., 2009).

The studies of Gerstner et al. used Laser Scanning Cytometry (LSC) aiming to define the DNA-ploidy as a marker of malignancy. LSC is automated and thus reproducible, but at least the equipment is cost-intense. A manual count is low cost but tedious, time-consuming and its results are not reproducible. To this end the authors of the present study aimed to combine an automated analysis that is less expensive. The authors hypothesized that the morphological features yield information about the malignancy/ benignancy of the respective sample.

The aim of the present study was to analyze light microscopic images of cytopathologic samples of thyroid cells using free software. The classification (diagnosis) was aimed to be conducted by artificial intelligence after a process of machine learning.

Samples of thyroid were chosen because the incidence of diagnosed thyroid cancers is increasing at least in Brazil and the USA. At least in those two countries, thyroid cancer is the fifth most frequent cancer in women. As stated in the recent scientific literature, an inexpensive but reliable methodology will always be an important issue to lower the costs for reliable medical diagnosis (Gerstner et al., 2002; Mittag et al., 2006; Gerstner et al., 2009; Buzin et al., 2015; Laffers et al., 2006; Melzer et al., 2016).

II. Materials and Methods

a) Study Design

Manual diagnostic data from cytopathological aspirates were provided by Cassiano Antonio Moraes University Hospital. The automated analysis was conducted at the Image Cytometry Laboratory 10 (B) at the Biopractices Campus of Vila Velha University. The type of study was cross-sectional where the observation of data will occur within a time interval, without the need for direct interaction with the patients.

b) Obtaining of Samples and Images

In the present study 52 samples submitted to fine needle aspiration stained by pap smear were collected with a diagnosis made by the pathologist Dr. Carlos Musso; selected from March/2021 to March/2022. The photos taken by the pathologist

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himself were collected and sent to the researcher. The study was previously approved by the ethics committee of the institution Vila Velha University - (No. 4.094.607) and of the institution Cassiano Antonio Moraes University Hospital - (No. 5.073.110).

c) Image Preparation and Analysis

The images have been saved in .tif format. Samples of each patient were stored in individual folders without personal identification. The received images were analyzed and classified using free software. The software chosen for nuclei analysis was CellProfiler (CP) (version 4.2.4), after the pipeline (Figure 1) was created in this software, the images of each patient were loaded, there was a background inversion where the colors were inverted to grayscale scales for processing the pixels, presenting you light objects on a dark background, identification of all objects (nuclei) and finally filtered (Figure 2 and 3), in order to exclude non-cellular artifacts.

Pipeline Used with Cellprofiler for All Samples

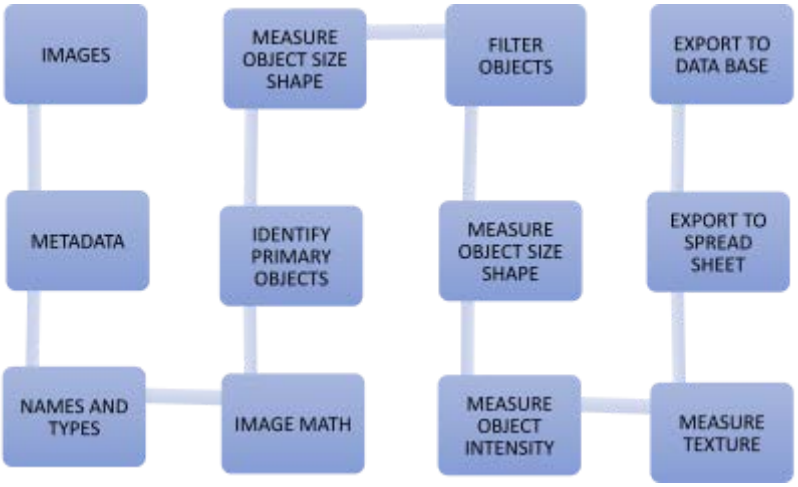


Figure 1: Pipeline: First all the images were loaded, identified by the unique format known in all .tif, the intensities were inverted for the objects of interest asking for light on a dark background, later the primary object was identified as nuclei 1, after filtering the objects (eliminating objects unexpected) a new count of the parameters was performed and then data nuclei 2 was found, only these were exported to carry out the classification.

Steps Using Cellprofiler – Malignant Sample

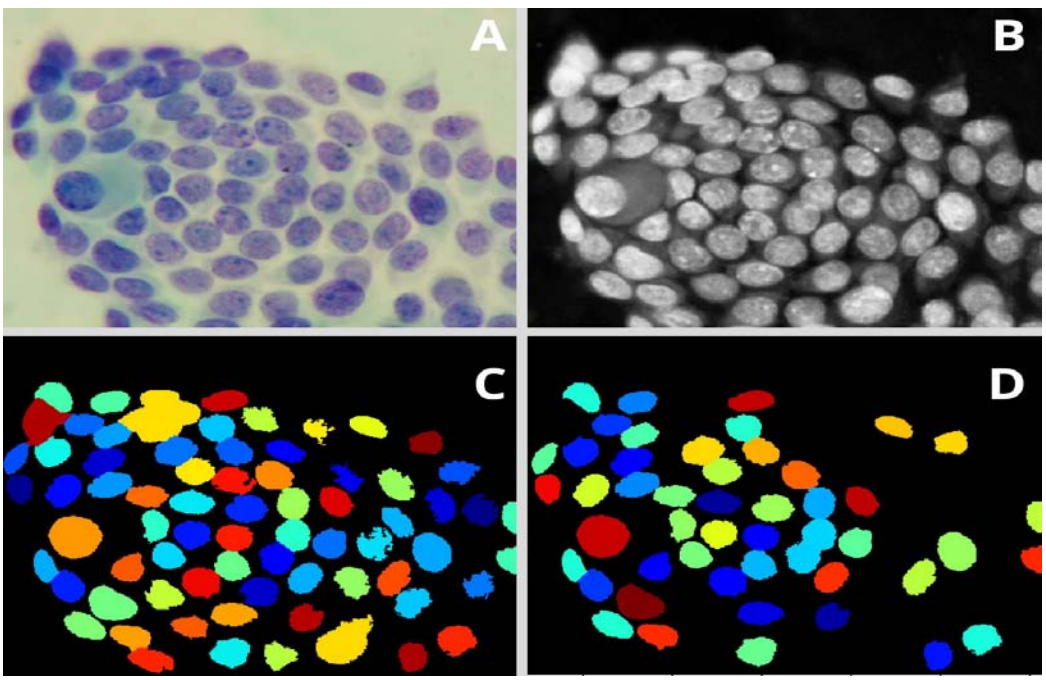


Figure 2: Analysis performed in Cellprofiler of a malignant sample. A: Original Image, B: Color inversion to grayscale, C: Nuclei identification D: Filter applied to exclude the artifacts.

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Steps Using Cellprofiler – Benignant Sample

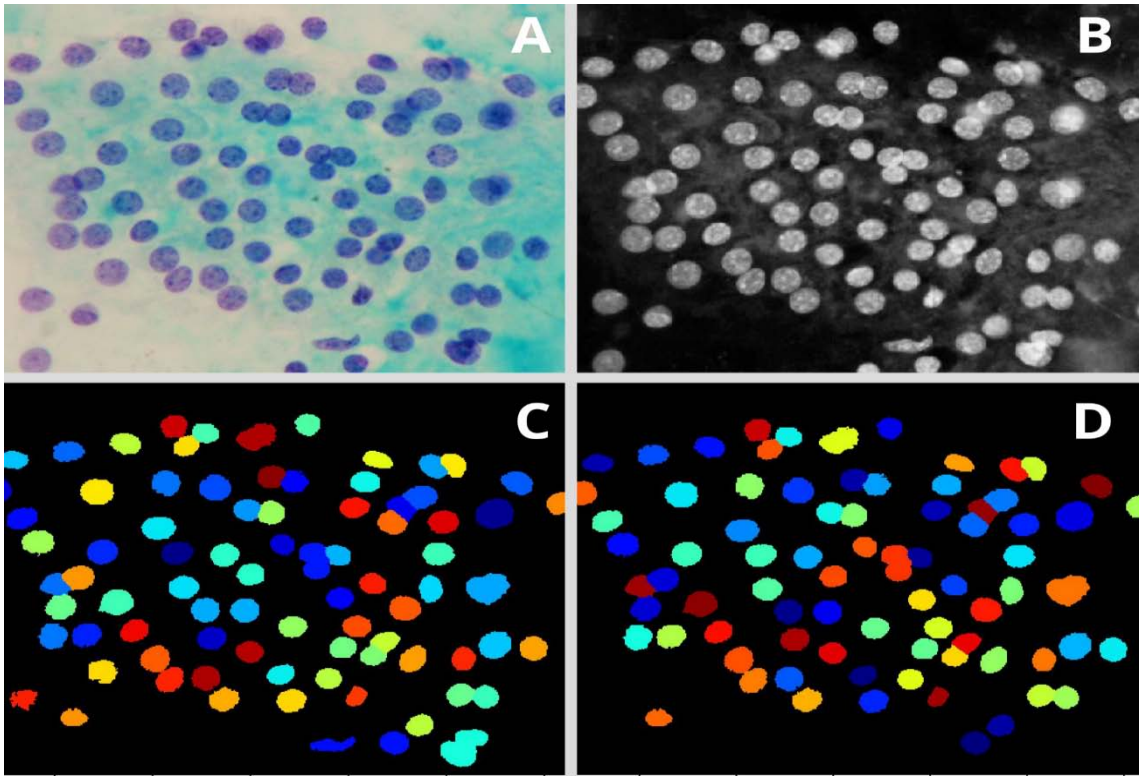


Figure 3: Analysis performed in Cellprofiler of a benignant sample. A: Original Image, B: Color inversion to grayscale, C: Nuclei identification D: Filter applied to exclude the artifacts.

The dates of the analyzed nuclei were exported to an excel file and later to another two software that performed the classification by supervised learning (Cellprofiler Analyst (CPA) and Tanagra (TG) software).

d) Statistical Analysis

After the complete analysis of all samples a comparison of the results of the effectiveness of the automated method and the manual diagnosis was made with MedCalc software (https://www.medcalc.org/download/). Also, calculations for specificity and sensitivity with CPA and TG software.

III. Results

The ROC curve (Figure 4) shows the progress of machine learning (AUC = 0.96), and the capability to differentiate the positive and negative.

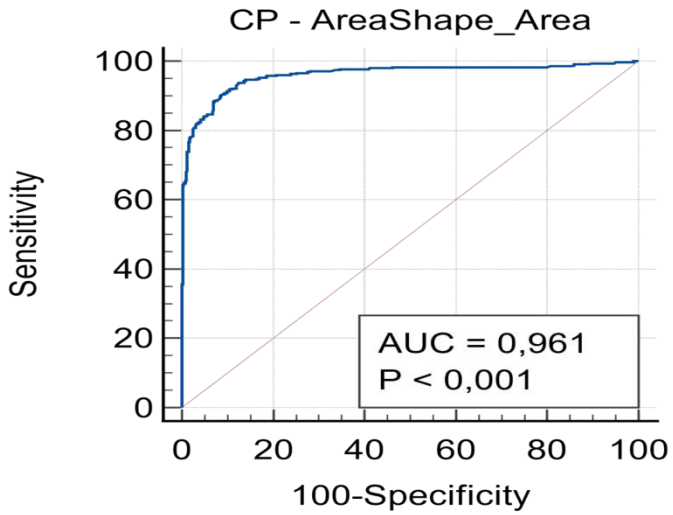


Figure 4: ROC curve between automated and manual results.

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TG is a macro to be integrated in excel for classification purposes, amongst others. If, like in the present study there are multiparametric data, TG offers the possibility to choose the best parameters for a successful classification (data mining), that is, parameters that do not substantially enhance the results will be excluded in this step.

The scatterplot shown in figure 5 concluded that the software was able to correctly differentiate the classes.

Scatterplot with Software Tanagra – Supervised Learning

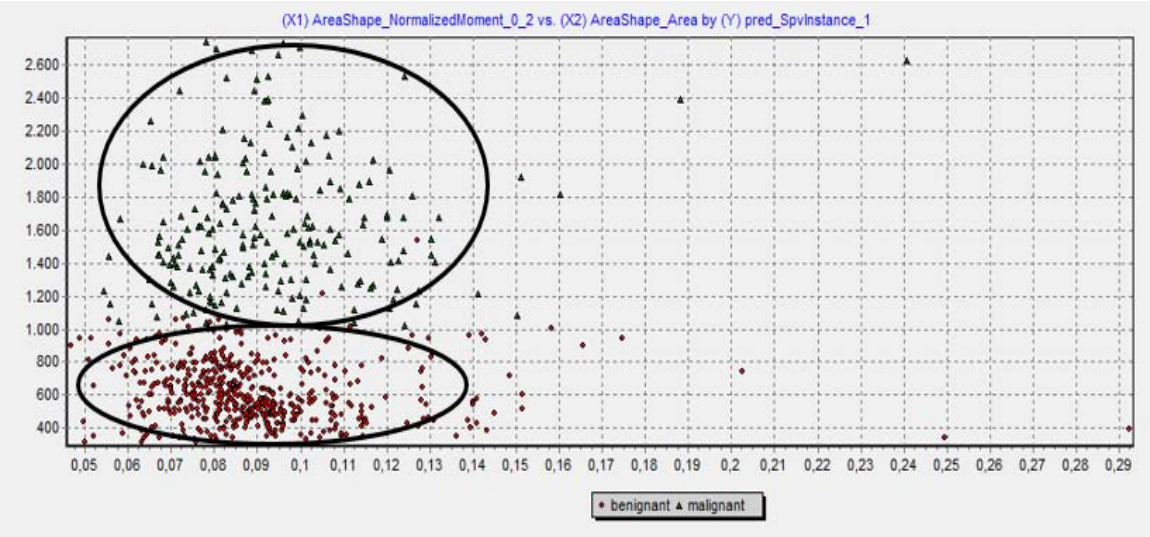


Figure 5: Classification obtained in the TANAGRA software (Scatter plot: red-benign / green-malignant).

The algorithm chosen by the authors was the Linear discriminant analysis (LDA). LDA is strongly related to analysis of variance (ANOVA) and regression analysis, which also attempt to express a dependent variable as a linear combination of other characteristics (Venkataraman et al., 2007). TG software presented data with high sensitivity (0.94) and specificity (0.91) (Figure 6).

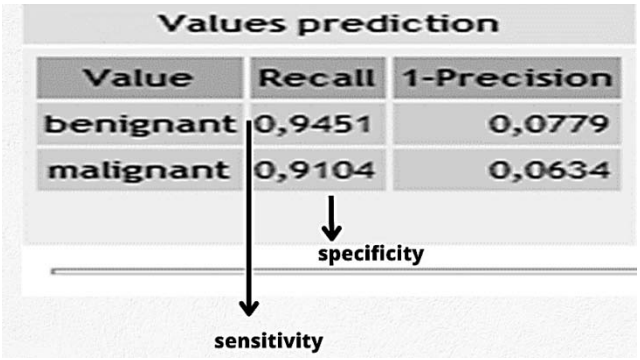


Figure 6: Linear discriminant analysis between benignant and malignant objects.

The other software used to also classify the data was CPA, which also received the same data calculated by TG software. Figure 7 represents the training manual classification made. CPA also supervised machine learning and the algorithm used was logistic regression. In this software the results were comparable to the TG software and also showed high performance in learning – 97.08% of accuracy.

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Cellprofiler Analyst Training

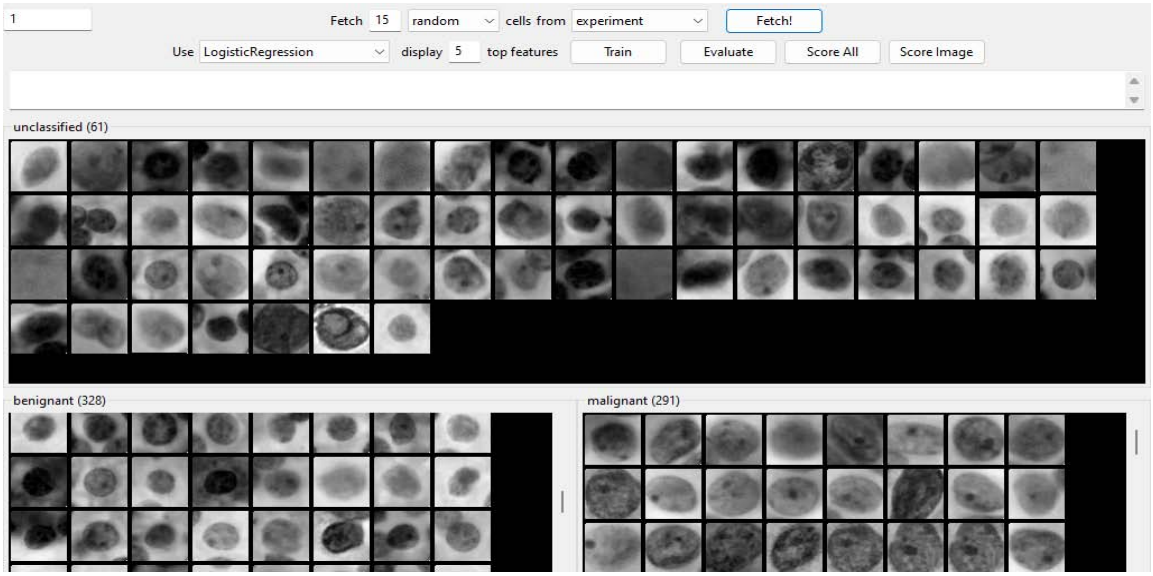


Figure 7: Training with two classes, malignant (lower right) and benign (lower left) cells.

After training benignant and malignant objects were requested to verify software performance to calculate sensitivity and specificity. (Figure 8 and 9).

Cellprofiler Analyst Performance - Benignant

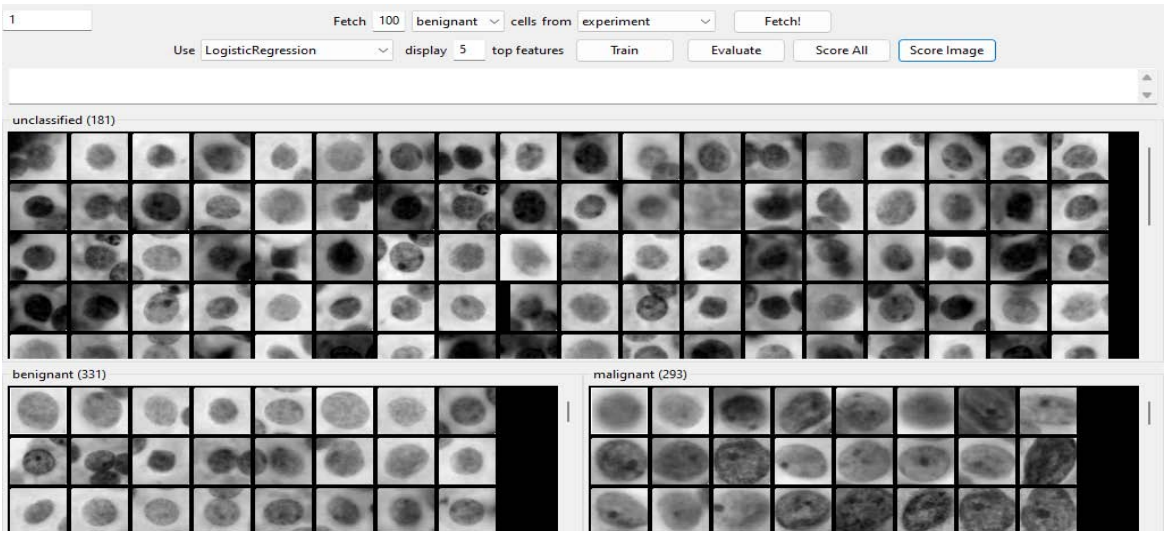


Figure 8: 100 benignant correct objects requested after training.

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Cellprofiler Analyst Performance - Malignant

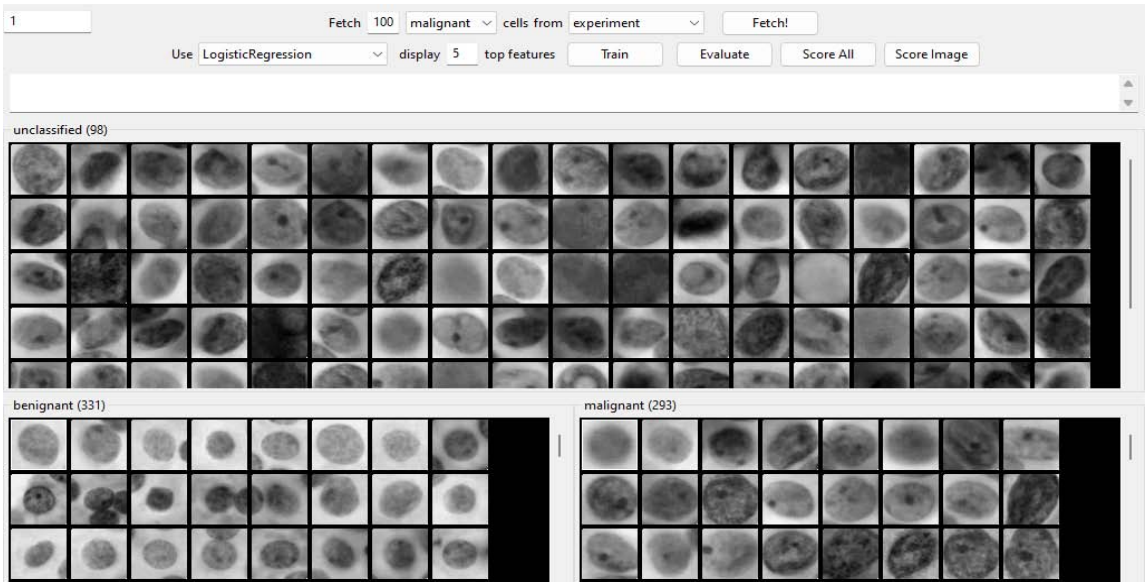


Figure 9: 100 malignant correct objects requested after training.

To check the machine learning progress, a score image tool is displayed after training to show the correctness of the machine (Figure 10).

Score Image of Malignant and Bengnant Nucleus – Cellprofiler Analyst

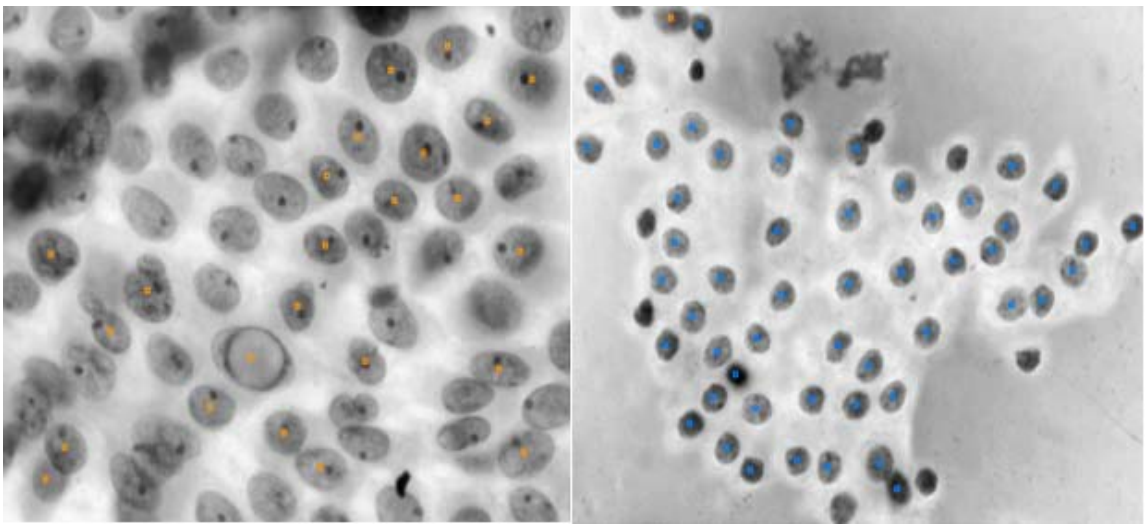


Figure 10: Score Image: This tool allows the analyst to see the full extent of the image and the objects there identified according to the classification performed under each class after training. A: Malignant objects identified B: Benignant objects identified. - 97.08% of accuracy.

The results calculated by CPA and TG obtained accuracy above 90%. Both software were able to distinguish the difference between classes. Regardless of the number of samples, the algorithm is able to learn and perform the correct classification of objects, as shown in the results of the present study.

IV. Discussion

Fine-needle aspiration cytology is a routinely used, reliable, quick, highly reproducible, minimally invasive, and cost-effective method for diagnosing nodular lesions of the thyroid (Rosai, 1996). It has been suggested as a first step test in the diagnosis of thyroid nodules (Segev et al., 2003; Aguilar et al., 1997). The most important contribution of this method to the diagnostic process is its power to distinguish patients who need surgical intervention from those who do not (Koss, 1992). Manual diagnosis, however, is time-consuming and not reproducible.

Currently there are free software where it is possible to view all objects and classify them with the best parameters. After training all objects, the software

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can even independently predict a correct diagnosis in new objects. Other studies also had satisfying results with data found in automated analysis of cellular objects (Deka et al., 2017).

An interesting study combining machine learning and papillary thyroid carcinoma published in 2021 showed several machine learning models were built to predict disease recurrence and all models had a confirmed accuracy of 90% or more (Park & Lee, 2021).

Automated quantitative analysis of cellular objects is currently considered the viable option in developed countries, because the scientific community proves that human-machine interaction has the potential to reduce human effort and increase confidence in algorithmic decision to solve clinical diagnoses (Jayatilake & Ganegoda, 2021; Sarker, 2021).

In the literature, there are few studies with images of the cell classification process (Wang et al., 2010; Wang et al., 2019), unlike this study, which the objective was to show step by step.

In terms of DNA ploidy there may be a low percentage of false positive artifacts. However, those may not have influenced the results of the present study as proven by the high accuracy of the results (Gerstner et al., 2006). The ROC curve was created only using the area of the nuclei, concluding the area of the nuclei is referring to the amount of DNA, which makes an analysis of the ploidy relatively easy, even when using light microscopy as in the present study.

The term digital pathology is considered as the future of histopathology. The authors agree that the integration of images and digital results is not yet standard, but it will significantly contribute to receiving digital pathology as part of the clinical evaluation (Athelogou & Huss, 2018; Ribeiro et al., 2019). Other studies concluded that after several highly accurate and scientifically proven studies, histopathology is ready for automation (Ribeiro et al., 2020, Lucas et al., 2021).

The main purpose of this study was to compare the diagnosis manual analysis (gold standard) and automated analysis. The results are comparable to the gold standard with high specificity and high sensitivity in two different classifiers.

Conflicts of Interest

Nothing to declare.

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Study of α-Amylase Inhibitory Activity of Fractions Purified by Chromatographic Methods of the Endophytic Fungus *Penicillium Brevicaule Alba-CC200*

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Abstract- In this study, the antidiabetic activity of purified fractions of the endophytic fungus Penicillium brevicaule alba-CC200 isolated from the plant Celosia cristata was investigated. Antidiabetic activity was evaluated by inhibition of α-amylase enzyme. The secondary metabolites of P. brevicaule alba-CC200 were partially purified by column chromatography and obtained fraction II inhibited α-amylase by 91.2%. High inhibitory activity was observed in fraction II and was determined to be cardiac glycosides and terpenoids by thin layer chromatography (TLC) and qualitative analysis. This requires a more precise study of the purified fractions using high-precision chromatographic methods.

Keywords: a-amylase, a-glucosidase, column chromatography, TLC, cardiac glycosides, terpenoids.

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Study of α-Amylase Inhibitory Activity of Fractions Purified by Chromatographic Methods of the Endophytic Fungus *Penicillium Brevicaule Alba-CC200*

Mukhammedov Iqbol [α], Gulyamova Tashkhan [σ], Ruzieva Dilaram [ρ], Nasmetova Saodat [ω] & Turdimuhammadova Sarvinoz [¥]

Abstract- In this study, the antidiabetic activity of purified fractions of the endophytic fungus *Penicillium brevicaule alba-CC200* isolated from the plant *Celosia cristata* was investigated. Antidiabetic activity was evaluated by inhibition of α-amylase enzyme. The secondary metabolites of *P. brevicaule alba-CC200* were partially purified by column chromatography and obtained fraction II inhibited α-amylase by 91.2%. High inhibitory activity was observed in fraction II and was determined to be cardiac glycosides and terpenoids by thin layer chromatography (TLC) and qualitative analysis. This requires a more precise study of the purified fractions using high-precision chromatographic methods.

Keywords: a-amylase, a-glucosidase, column chromatography, TLC, cardiac glycosides, terpenoids.

I. Introduction

According to the World Health Organization, there are more than 400 million diabetic patients in the world today. According to analysis, this figure may exceed 550 million by 2030. In Uzbekistan, 220,000 people have been registered with this disease [1]. In recent years, diabetes has taken the third place among the causes of death, and cardiovascular and oncological diseases are in the second place. The social consequences of diabetes are also negative, because the treatment of this disease requires huge costs from the health care system [2]. Diabetes mellitus is a disease characterized by the presence of chronic hyperglycemia accompanied by metabolic disorders of carbohydrates, lipids and proteins [3].

In the last decade, a number of new methods of diabetes treatment have been proposed, but three areas remain the main ones: a) diet, insulin for patients with type 1 diabetes, b) hypoglycemic drugs used for type 2 diabetes, c) general procedure and physical activity [2].

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There are many different therapeutic approaches in the treatment of type 2 diabetes. Inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase is one of the important approaches to lowering postprandial blood glucose levels. Such inhibitors, which are used in clinical practice for the treatment of diabetes mellitus, are known to be associated with various adverse gastrointestinal effects. Therefore, it is necessary to identify amylase inhibitors from natural sources with less adverse effects. *Caesalpinia bonducella* aqueous extract inhibited α-amylase activity by 87.26% [3]. The inhibitory effect on α-amylase was studied by ethanol extract of *Capparis spinosa*. A concentration of 25 mg/ml of extracts from roots and leaves inhibited α-amylase by 97.31-98.92% [4].

In vitro α-amylase inhibitory activity of secondary metabolites isolated from *S. persica* and the chemical composition responsible for its activity were studied. Aqueous extract of *S. persica* highly inhibited α-amylase activity by 72.39% and had an IC_{50} value of 376 μg/ml compared to acarbose with an inhibitory value of 65.99%. In the analysis of TLC, it was found that there are 15 different compounds [5].

Albidopyroneis synthesized by *Streptomyces sp.,* an actinomycete isolated from sediments at the bottom of the Atlantic Ocean. The chemical structure of albidopyrone is a six-membered aromatic lactone, with a methyl group in the 3rd position, an OH group in the 4th position, and an acetic acid aniline amide residue in the 6th position. Albidopyrone is not an antibiotic, but together with tyrosine kinases, it inhibits the activity of tyrosine phosphate B (TPB), which regulates tyrosine phosphorylation. TPB is the main negative regulator of insulin (controls the phosphor state of the insulin receptor). In this regard, TPB inhibitors are effective agents for the treatment of diabetes [6].

Botryodiplodia teobromae is an endophytic fungus isolated from the weed *Euphorbia hirta*. Fungi were treated with 1mM $AgNO_3$ to synthesize silver nanoparticles. The production of silver nanoparticles was confirmed using the cell filter and surface color

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change from colorless to dark brown. Silver nanoparticles synthesized from *Botryodiplodia theobromae* fungi show the highest inhibitory percentage (94%) for α-amylase [7]. Endophytic fungi were isolated from the leaves of *Artocarpus heterophyllus*. The inhibitory activity of the obtained extracts on α-amylase was 89%, and 71% on standard acarbose [8].

In the treatment of patients with type 2 diabetes, α-glucosidase enzyme inhibitors are used. One of them, acarbose, is a pseudo tetrasaccharide that competes with mono- and disaccharides for digestive enzyme binding sites and slows the sequential fermentation of carbohydrates and absorption of carbohydrates in the small intestine [2, 4]. Butacarbose is mainly known as an α-glucosidase inhibitor and causes side effects such as abdominal distension, flatulence, and diarrhea [9, 10].

In pharmaceutics, natural products provide greater structural diversity than standard synthetic substances, offering great opportunities for the discovery of new compounds [11]. Lowering blood glucose is an effective tool in improving the survival rate of patients with diabetes. Endophytic fungi isolated from plants can be an alternative source of secondary metabolites for the treatment of diabetes [12].

Endophytic fungi are a hidden treasure trove of chemically important compounds with potential biological activity. Many natural compounds have been isolated from various endophytes [13]. Bioactive compounds produced by endophytic fungi include alkaloids, benzopyranones, quinones, cytochalazins, depsipeptides, enniatins, flavonoids, furandiones, isocoumarins, peptides, polyketones, phenols, hydroquinones, terpenoids, tetralones, and xanthones. Therefore, endophytes constitute a chemical reserve of new biologically active compounds, which are used in the pharmaceutical and agrochemical industries as antioxidant, antimicrobial, antidiabetic, antiparasitic, antiviral, insecticidal, antibiotic, immunosuppressive, and immunomodulatory agents [14, 15]. Endophytic fungi produce a variety of chemicals to allow them to live in plant cells. Due to their symbiotic relationship, they secrete pharmaceutically important biologically active compounds and enzyme inhibitors. 32 endophytes were isolated from the *Gymnema sylvestre* plant and their inhibition of α-amylase and α-glucosidase of *Fusarium equiseti* endophytic fungus was observed [16]. Asan α-amylase inhibitor, the IC_{50} value of the methanol extract of *Penicillium oxalicum* was 46.73 mg/ml, and the chloroform extract was 59.20 mg/ml [17]. Extracts from the endophytic fungus *Aspergillus awamori* isolated from *Acacia nilotica* inhibited α-amylase by 81% and α-glucosidase by 80% [18].

Secondary metabolites of the endophytic fungus *P. brevicaule alba-CC200* isolated from *Celosia cristata* were studied as inhibitors of pancreatic α-amylase enzyme. The ethylacetate extract of the obtained secondary metabolites showed 93% high inhibitory activity to α-amylase, and qualitative analysis revealed that the extract contained terpenoid, saponin, glycoside and phenolic components [19]. At the same time, to confirm which of the detected substances exert inhibitory activity, the method consists of elucidating through TLC, column chromatography and qualitative analysis methods.

II. Material and Methods

a) Cultivation of endophytic fungi

Both endophytic fungi were grown in the Czapek Dox nutrient medium on orbital shaker Elon 357 (Joanlab, Poland) at 180 rpm, 28°C, and for seven days. The biomass of the cultures was yielded by centrifugation at RS-6 (RHSnab, Russia) at 6,000 rpm and stored at +4°C.

b) Determination of the inhibitory activity

Each sample obtained after the separation of the methanol fraction on the column was examined for inhibitory activity. The activity of the α-amylase fractions was determined according to the method used in plant extracts [20]. The starch solution prepared as a substrate in an amount of 1 g/10 ml of water, boiled for 2 min, the sample volume adjusted to 100 ml by distilled water. 100 ml of pancreatic α-amylase (0.1 M Na-acetate buffer is 13 ml at pH 7.2), 100 mcg of endophyte extract, 2 ml of acetate buffer were incubated for 10 minutes at 30° C for 2 ml of starch prepared from the preparation. The incubation reaction was then stopped and immersed in 10 ml of an aqueous reagent, and the optical density was measured at 630 nm on a SPECOL-1300. To prepare the iodine reagent, 0.5 g of crystalline iodine, 5 g of potassium iodide, and 250 ml of dissolved in water were taken; 2 ml of this reagent was added to 100 ml of 0.1 M HCl to obtain a working solution. The inhibitory activity was expressed by the formula: (A0-At)/A0x100%, where A0 is the absorption of the control sample, and T is the absorption of the experimental sample, respectively. As a comparison drug, acarbose from the commercial drug "Glucobay" (Bayer Pharma AG, Germany) was used.

c) Phytochemical analysis of extracts of secondary metabolites

Qualitative composition of compounds in extracts of endophytic fungi determined according to Prabhavathi et al. [21].

The tannins and phenolic substances was determined by adding 2-3 drops of 1% $FeCl_3$ solution to 2 ml of the extract. In the presence of iron ions, tannins give a black-blue or black-green color, and phenols - are purple.

The presence of saponins was established by diluting 1 ml of the extract with 5 ml of hot water (60 °C)

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with intensive shaking for 5 minutes until the formation of a persistent foam. The foam volume was maintained for the next 30 minutes.

The terpenoids were determined by mixing 0.5 ml of the extract with 2 ml chloroform and 3 ml of H_2SO_4 (conc.). The formation between the phases of red-brown staining indicates the presence of terpenoids. 2 ml of the extract was mixed with 4 ml of hexane and shaken to determine the terpenoids. At the same time, the separation of the extract into 2 layers was observed. The upper layer was separated, 4 ml of 10% ammonia was added, and the lower layer's color was determined. The purple-pink color indicated the presence of anthraquinones.

The presence of cardiac glycosides was determined by mixing 1 ml of the extract with 1 ml of glacial acetic acid and then adding one drop of 3% ferric chloride in methanol. Then H_2SO_4 (conc.) was added along the tube wall, and the color of the lower layer was determined. Blue-green staining indicated the presence of cardiac glycosides.

To determine flavonoids, a few drops of 20% sodium hydroxide were added to 2 ml of each extract, and the formation of an intense yellow color was observed. Next, a few drops of 70% dilute hydrochloric acid was added, and the yellow color disappeared. The formation and disappearance of yellow color indicate the presence of flavonoids in the sample extract.

The alkaloids was determined by their ability to form compounds insoluble in water with complex iodides, which makes it possible to establish the presence of alkaloids even with their insignificant content. A solution of iodine in potassium iodide (Wagner reagent, Bouchard reagent) with alkaloids form brown, hardly soluble in water precipitates. Five drops of the reagent for precipitation of alkaloids are added to 1 ml of the extract. In the presence of alkaloids, a brown precipitate appears.

d) Thin layer chromatography (TLC)

In thin-layer chromatography, components are partitioned between stationary and mobile phases. Separation of the constituent parts moves at different speeds in the TLC plates depending on its polarity in stationary and mobile phases. SIGMA-ALDRICH Silicagelon TLCAL plates of size 10x10 cm were used for TLC. Plates were run in the following eluents: benzene: methanol (40:8) in a standard chromatographic chamber. 25µl of the extracts at a concentration of 25 mg/ml were dropped onto the starting line of the plates. The plates taken from the chromatographic chamber were dried and the following methods were used to determine the separated substances: under ultraviolet light at 254 nm; with iodine vapor in a desiccator; 20% solution of phosphorous-molybdic acid in alcohol; It was sprayed with a 10% solution of H_2SO_4 acid in methanol and dried at 110°C.

e) Column chromatography

Partial purification of the extracts was performed with modifications from Kaur et al. The extract was applied to a column (2 × 25 cm) of silica gel (size 100–250). The mobile phase used is chloroform: ethyl acetate: formic acid in a ratio of 5:4:1. Using this solvent system, fractions of 10 mL each were collected and designated as I and II, respectively. Activity was observed in fraction II. The chemical nature of the active fractions was determined using qualitative analysis and TLC methods [22].

Results were the mean of replicate analysis.

III. Results and Discussion

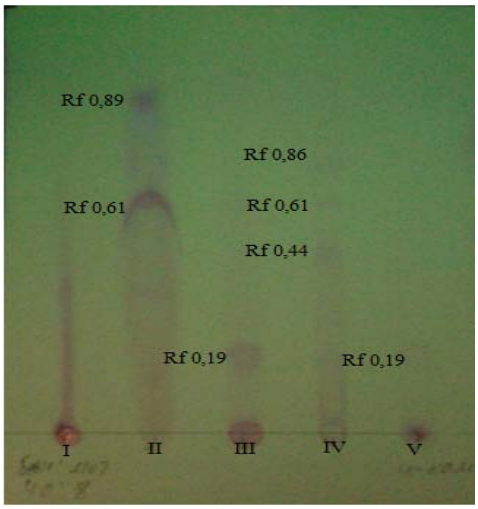
58 compounds that can inhibit α-amylase and α-glycosidase enzymes were identified in hyperglycemia. These substances mainly belong to flavones, flavone glycosides, triterpenes, alkaloids, tannins and other polyphenol compounds [23]. Partially purified enzyme inhibitors result in high activity, with maximal inhibitory activity determined based on morphological and molecular analysis [24].

Our results show that ethylacetate extract of *P. brevicaule alba–CC200* contains compounds responsible for the inhibition of amylase activity, so the extract was purified. Five fractions were obtained from the extracts by column chromatography, dissolved in DMSO and tested as an α-amylase inhibitor. From the data in Table 1, it can be seen that the highest inhibitory activity was 91.2% in fraction II, and the methanol fraction before chromatographic purification was 88.7%. Low inhibitory activity was 58.4% in fraction I, 26% in fraction III, 38.7% in fraction IV, and 12.4% in fraction V.

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Table 1: Analysis of the inhibitory level of secondary metabolites of the endophytic fungus *P. brevicaule alba-CC200* and the analysis of fractions obtained by column chromatography.

Fractions	α-amylase % inhibitor	Rf
I	58,4	-
II	91,2	0,89
		0,61
III	26	0,19
IV	38,7	0,86
		0,61
		0,44
V	12,4	0,19



Eluent system: benzene:methanol (5:1). The volume of samples used was 25 µl. Determining reagent: 20% alcoholic solution of phosphorus-molybdic acid.

Fractions obtained as a result of column chromatography were checked for chemical purity of substances using TLC. When TLC plates were treated with a 20% alcoholic solution of phosphoro-molybdic acid, the spots were colored blue, which is due to the presence of sopanin and triterpenes. Jasleen Kaur et al., partially purified *Alternaria* endophytic fungus and when treated with $FeCl_3$ on TLC plates, the spots were stained purple, which can be attributed to the characteristic of phenolic compounds in the fraction [25]. Partially purified fraction III obtained from the endophytic fungus *Colletotrichum capsici* was found to have potent antidiabetic activity. In addition, determination by mass spectrometry showed that compounds in fraction III are mainly fatty acid and phenolic compounds [26]. Terpenoids, formaldehyde and eugenol biologically active substances were detected when ethylacetate extracts of endophytic bacteria isolated from *Mentha piperita* (mint) plant were examined using GC-MS spectrum [27]. Antioxidant, antidiabetic and anticholinesterase activity of biologically important phytochemicals contained in the methanol extract of *C. uredinicola* endophytic fungi was determined in vitro. The qualitative analysis of *C. uredinicola* extract shows the presence of flavonoids, tannins, alkaloids, glycosides, phenols, terpenoids and coumarins [28].

P. brevicale alba-CC200			
Fraction	α-amylase % inhibitor	Terpenoids	Cardiac glycosides
II	91,2		

Figure 1: Qualitative analysis of fraction II obtained by column chromatography

Also, fraction II with high inhibitory activity was tested by qualitative reactions for several chemical classes. The presence of cardiac glycosides and terpenoids was determined in the fraction with 91.2% α-amylase inhibitor. Figure 1.

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Many studies suggest the use of triterpenes in the prevention of diabetic complications such as nephropathy, embryopathy, neuropathy or wound healing [29]. For example, oleanolic acid, betulinic acid and ursodeoxycholic acid are strong inhibitors of TGR5 receptors involved in energy metabolism [30]. The n-hexane fraction with the highest inhibitory activity of the plant *Nuxia oppositifolia* confirmed the presence of 3-oxolupenal and cathononic acid when examined using GC-MS analysis [31]. Many reports attribute the α-amylase inhibitory effect of the extracts to the terpenoids present in the extracts [32, 33].

IV. Conclusion

Fraction II of the endophytic fungus *P. brevicaule alba - CC200*, purified by column chromatography, inhibited α-amylase by 91.2%. TLC and quality analysis showed that the high inhibitory activity of fraction II is due to the content of cardiac glycosides and terpenoids. These natural bioactive compounds can inhibit α-amylase enzyme activity and regulate blood glucose levels. It can be an alternative source for hyperglycemic compounds.

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Fellows/Associates of Medical Research Council

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23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.



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Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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