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acknowledgement sections. Title, abstract and key words should be written in both Turkish and English. The manuscript should be formatted in accordance with the above-mentioned guidelines and should not exceed 16 A4 pages.

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Acknowledgements: Any technical or financial support or editorial contributions (statistical analysis, English/Turkish evaluation) towards the study should appear at the end of the article.

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Original Articles

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ORIGINAL ARTICLE

Smartphone Digital Image Colorimetry for the Determination of Aluminum in Antiperspirant Products

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ABSTRACT

Objectives: This study aims to present a method for the determination of the aluminum in antiperspirant products (APPs) by chelating it with quercetin before its detection by smartphone digital image colorimetry (SDIC).

Materials and Methods: Samples were prepared by closed-vessel acid digestion in PTFE cups. This was followed by complexation of aluminum in the sample solution using quercetin as a chelating agent. Sample solutions were transferred into a quartz ultraviolet/visible detection microcuvette for detection in a homemade colorimetric box designed for capturing images of the yellow complex with a smartphone camera. The pixel intensity of the images was converted to numbers for quantitation using ImageJ software for a personal computer. An independent study using high-performance liquid chromatography-diode-array detection was conducted to check the accuracy of the proposed method.

Results: Optimum SDIC conditions included a Samsung C9 smartphone as the detection camera, a cropped region of interest of 6400 px^2, and the side position of the colorimetric box were selected for capturing the images of the sample solutions placed 10.0 cm from the detection camera, whereas optimum complexation conditions were found to be as sample pH of 5.5, sample volume of 3.0 mL, complexation time of 1.0 min and a ligand concentration of 0.28 mmol L^{-1}. Analytical performance of the method included a limit of detection of 0.5 µmol L^{-1} and a coefficient of determination (R^2) of the calibration graph of 0.9981.

Conclusion: The proposed method was successfully applied for the determination of aluminum in APPs with percentage recoveries ranging from 80.0 to 109.6%.

Key words: Aluminum, antiperspirant products, digital image colorimetry, quercetin, smartphone

INTRODUCTION

Aluminum and its compounds are widely used in various industries, including food, cosmetics, and pharmaceuticals.[1] Personal care products, such as deodorants and antiperspirant products (APPs), block the unpleasant body odor and reduce underarm sweat. They contain ingredients for pleasant odor, skin care, and beauty.[2] The main active ingredients in APPs are aluminum and zirconium compounds.[3] According to some studies, aluminum compounds can accumulate in the human body and are suspected of contributing to various pathological disorders including anemia, bone disease, breast cancer, encephalopathy, Alzheimer's, Parkinson's, and other neurological disorders.[4,5] Despite the ongoing debate over the use of aluminum in APPs and the fact that the concentration of aluminum compounds in the final product is rarely reported, the number of analytical methods proposed for their determination in these samples remains very limited. Consequently, it is imperative to develop straightforward, rapid, and inexpensive analytical methods for this purpose to benefit consumers as well as governmental health agencies during the legislative process.

This work was partially presented at the 2nd International Congress on Analytical and Bioanalytical Chemistry (2nd ICABC 11-14 March 2020) Antalya, Türkiye.

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Although flame-atomic absorption spectrometry (FAAS) is the most commonly used technique for elemental determination, graphite furnace-atomic absorption spectrometry (GFAAS)[6] is preferred for aluminum, being a refractory element, due to the former's poor atomization efficiency and thus low sensitivity. Nonetheless, GFAAS is expensive, requires extensive experience to generate reproducible results, and is not available in small analytical laboratories. The techniques of choice for determining aluminum and its compounds in personal care products, including APPs, are high-performance liquid chromatography with ultraviolet/visible detection (HPLC-UV/Vis),[7,8] flow injection-UV/Vis spectrophotometry,[9] and flow-through potentiometry.[10] Despite their numerous benefits, the high operating costs and high level of expertise required to use them make it difficult for small laboratories to purchase and use them efficiently. Furthermore, these instruments are electricity-dependent and not designed for on-field analysis, necessitating the development of alternative techniques. Miniaturized detection techniques that overcome these limitations have recently received special attention. A colorimetric solid-phase extraction method was proposed for the determination of aluminum, in which a membrane disk loaded with the colorimetric reagent pyridoxal salicyloylhydrazone allowed the extraction of the metal complex before its detection using a miniature fiber optic spectrometer.[11] To detect the aluminum complex in the visible region, a paper platform with alizarin S as a chromogenic reagent in a spot test was used in conjunction with diffuse reflectance spectroscopy using a portable spectrophotometer.[12]

Digital image colorimetry (DIC) is a new colorimetric technique that utilizes the basic red-green-blue (RGB) channels of images of a colored analyte solution captured by a digital camera, webcam, hand scanner, or smartphone camera.[13,14] Smartphones are the best image acquisition tools for this purpose because of their superior photography functions, software, and portability for image processing. Smartphone DIC (SDIC) has been applied for the determination of total iron,[15] iron (II),[16] chromium (VI),[17] arsenic (III),[18] cobalt (II)[19] and boron[20] in addition to several organic analytes in various samples. To the best of our knowledge, this is the first study on the use of SDIC for the determination of aluminum.

In this study, an SDIC method is proposed for the determination of aluminum in APP samples after chelating it with quercetin to form a yellow complex that can be easily detected by a smartphone camera. The results of SDIC were compared with HPLC-diode-array detection (HPLC-DAD) method for accuracy check.

MATERIALS AND METHODS

Chemicals and reagents

All the reagents used were of analytical grade unless otherwise indicated. Acetic acid, HPLC-grade acetonitrile, aluminum nitrate nonahydrate, disodium phosphate, ethanol, hydrochloric acid, monosodium phosphate, nitric acid, quercetin, and sodium acetate were obtained from Sigma-Aldrich (Steinheim, Germany), while trifluoroacetic acid was obtained from Fluka (Buchs, Germany). Deionized (DI) water (18.2 MΩ-cm), purified with Pure Lab Ultra Analytic (ELGA LabWater, High Wycombe, UK), was used for the preparation of all aqueous solutions.

Instrumentation

A homemade colorimetric box with the dimensions of 20 × 15 × 8 cm^3 was used. The white interior walls provided uniform illumination. Sample solutions were transferred into a quartz UV/Vis microcuvette (Hellma, Kruibeke, Belgium) and placed inside the box 10.0 cm from the detection camera and 11.0 cm from a 1.2V/1300 mA battery-powered white light-emitting diode that illuminated the sample solution. Images were captured with the flash being turned off. A diagram of the colorimetric box is shown in Figure 1. Digital images were captured using an SM-C9 smartphone, equipped with a 16 MP rear camera with a resolution of 4608 × 3456 pixels. Images were processed using

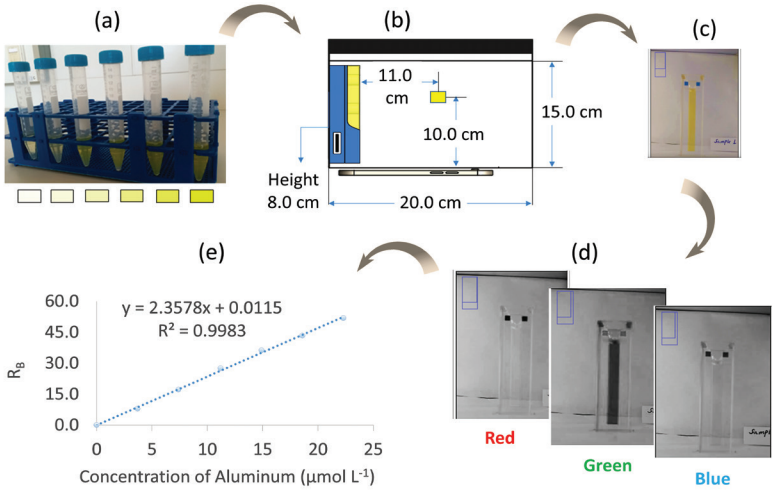

Figure 1. Proposed smartphone digital image colorimetry system: (a) Solution of aluminum-quercetin complex at different concentrations; (b) colorimetric box; (c) image of sample; (d) image split into red-green-blue channels; and (e) external aqueous calibration graph; $R_B = 255\text{-}I_B$, where, R_B and I_B are the response and intensity of the analyte solution in the B channel, respectively

the freely downloadable software ImageJ (PC version, 1.52a, Java 1.8.0_212, 64 bit) from the National Institutes of Health, USA.[21] An HPLC instrument (1200 Series, Agilent Technologies, Santa Clara, CA, USA), equipped with a degasser, a quaternary pump, an autosampler, a column thermal jacket and a DAD was used for accuracy check of the method.

Standard solutions of aluminum

A 3.7 mmol L^{-1} stock solution of aluminum was prepared by dissolving an appropriate mass of aluminum nitrate nonahydrate in 0.10 mol L^{-1} hydrochloric acid solution, from which a 0.37 mmol L^{-1} intermediate standard solution was prepared in the same diluent. Working standard solutions were prepared in the ranges of 1.7-25.0 µmol L^{-1} for SDIC and 3.3-60 µmol L^{-1} for HPLC-DAD in the same diluent. A 3.3 mmol L^{-1} stock solution of quercetin was prepared by dissolving an appropriate mass of the solid in ethanol/DI water (60/40%, v/v). The pH was adjusted to 5.5 with a 1.0 mol L^{-1} acetate buffer solution.

Sample preparation

Five APP samples of various brands were obtained from local groceries and pharmacies in Nicosia, TRNC. The samples were digested using a previously developed method[22] with some modifications. Briefly, 0.25 ± 0.01 g of each sample was weighed and digested in 3.0 mL of aqua regia in PTFE cups for 2.0 h at 100°C in a closed-vessel digestion block. After allowing the samples to cool down to room temperature, they were filtered through a 0.22 µm Whatman filter paper into a 25.0 mL volumetric flask and the volume was completed to the mark with DI water. From this solution, 1.0 mL was transferred into another 25.0 mL volumetric flask and the volume was made up to the mark with DI water. This solution will be referred to as the sample solution henceforth.

Complexation reaction and smartphone digital image colorimetry

For complexation, 250 µL of the 3.3 mmol L^{-1} quercetin solution was added to 50.0 µL of the sample solution in a 15 mL screw capped graduated polypropylene centrifuge tube. The mixture was made up to 3.0 mL with acetate buffer solution (pH 5.5). After the solution was vortexed for 1.0 min, a portion (ca. 100 µL) was transferred into a quartz UV/Vis cuvette for SDIC analysis.

Chromatographic conditions

The aluminum-quercetin complex and the excess ligand were separated using a reversed-phase column (Agilent Eclipse XDB-C18 4.6 mm ID x 150 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA), a gradient elution consisting of 0.5% trifluoroacetic acid in DI water, pH 1.40 (solvent A) and acetonitrile (solvent B), with a gradient program of 10% solvent B at 0 min to 80% solvent B within 10 min at a flow rate of 1.0 mL min^{-1}. Separation temperature and the injection volume were set at 30°C and 20 µL, respectively. The maximum absorption wavelengths of the complex and ligand were 415.0 and 374.0 nm, respectively. The chromatograms obtained are given in Figure 2.

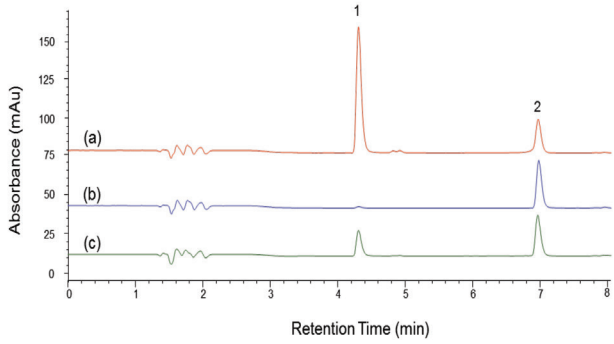

Figure 2. HPLC-DAD chromatograms: (a) Aluminum standard in 0.10 M hydrochloric acid (50 µM); (b) quercetin (100 µM); (c) antiperspirant product. Separation conditions: Column, Agilent Eclipse XDB-C18. 4.6 mm ID x 150 mm, 5 µm, gradient mobile phase with 0.5% trifluoroacetic acid in DI water, pH 1.4 (solvent A) and acetonitrile (solvent B), 10% B at 0 min to 80% B within 10 min at a flow rate of 1.0 mL min^{-1}, column temperature, 30°C, injection volume, 20 µL, detection wavelength, 415 nm. Peaks: 1, aluminum-quercetin complex, and 2, quercetin

HPLC: High-performance liquid chromatography, DAD: Diode-array detection

Statistical analysis

Upon splitting the images into their RGB channels, the mean histogram values from the B channel were used for calibration and quantitation throughout the analysis. For statistical analysis, a single-factor analysis of variance test (ANOVA) in Microsoft Office Excel (2013 Windows, Microsoft Corporation, WA, USA) was used; a *p* value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Optimization of smartphone digital image colorimetry conditions

Data processing

The mean value of each channel was obtained from its histogram and the B channel, giving the highest intensity, was used for the rest of the experiments. Because in the RGB model, the more intense the color, the lower the values within the range of 0-255, the value obtained from the histogram required additional processing to obtain a positive slope. Beer's Law, represented by the equation $R= log (I_0/I)$, where R is the response, I_0 is the mean intensity of the blank, and I is the mean intensity of the analyte, all measured from the same channel, has been used to process RGB data in the literature.[23] The equations $R= 255-I$,[24] and $R= I_0 - I_b$ were also used, where, I_0 and I_b are the mean intensity of the blank and the analyte solutions in the blue channel, respectively.[25] In this study, the data were processed using equation 1.

$$R_B= 225-I_B \qquad \text{equation 1}$$

where, R_B and I_B are the response and intensity of the analyte solution in the B channel, respectively.

Detection camera and region of interest

The overall quality of an image is influenced by several factors, such as resolution, aperture, specifications of the light sensor as well as the algorithm applied for image processing.[26] The effect of camera type on the response was assessed using four different phones, namely, SM-C9, Techno p-3, SM-S7, and iPad 1460 with camera resolutions of 16 MP, 13 MP, 12 MP, and 5 MP, respectively. The response increased as the resolution increased (Figure 3a), indicating that this parameter is important in determining the image quality. Therefore, SM-C9 was used for further analysis. The digital images captured for sample solutions were processed within a defined region of interest ranging from 400 to 14400 px^2. The results revealed no significant differences in the response, owing primarily to the homogeneity of the sample solutions (Figure 3b). An area of 6400 px^2 was used throughout the analysis.

Position of camera and cuvette

Compared to the side position, the top position produced blurred images and a slightly lower response (Figure 3c). As a result, the side position was used for further analysis. The effect of the distance between the sample solution and the detection camera was investigated over a range of 8.0-12.0 cm with the image blurring below 8.0 cm. The response remained nearly constant throughout the entire range under study (Figure 3d), indicating that the detection camera's maximum autofocus efficiency was achieved within this distance. For the following experiments, an optimal distance of 10.0 cm was chosen between the detection camera and the sample solution.

Optimization of complexation conditions

Sample pH

pH of the sample solution affects both the formation of a stable complex and the form of the analyte before complexation. Aluminum exists primarily in its trivalent form under acidic conditions, whereas increasing the pH leads to the formation of insoluble aluminum hydroxide at neutral pH.[8] The effect of pH on the analytical response was studied over a pH range of 4.5 to 6.5. The solution was colorless at pH less than 4.0 and an unstable yellow color formed at pH greater than 7.5. The response improved up to pH 5.5, beyond which, it declined and remained almost constant (Figure 4a). A similar result was obtained in a previous study for the complexation pH of aluminum with quercetin.[8] As a result, pH 5.5 was deemed optimal throughout the study.

Concentration of the ligand

To ensure that the maximum yield of the complexation reaction was achieved, the addition of an excessive amount of quercetin is necessary. The effect of the ligand concentration was carried out by using a fixed concentration of aluminum (0.31 mmol L^{-1}), in a 3.0 mL sample solution buffered at pH 5.5, and varying the concentration of quercetin from 0.06 to 0.50 mmol L^{-1}. The response increased with increasing the concentration of quercetin up to 0.28 mmol L^{-1}, after which it remained constant, indicating that the optimum molar ratio of metal to ligand was reached, which was in accordance with the theoretical stoichiometric molar ratio of 1:1 between aluminum and quercetin, as also found experimentally in a previous study.[7] Therefore, the optimum concentration of quercetin was taken as 0.28 mmol L^{-1} (Figure 4b).

Sample volume and complexation time

Sample volume affects the concentration of the aluminum-quercetin complex in the final solution. The effect of increasing the sample volume was investigated from 3.0 to 7.0 mL. Notably, 3.0 mL was the minimum sample volume suitable to obtain a buffered solution after acid digestion of the real

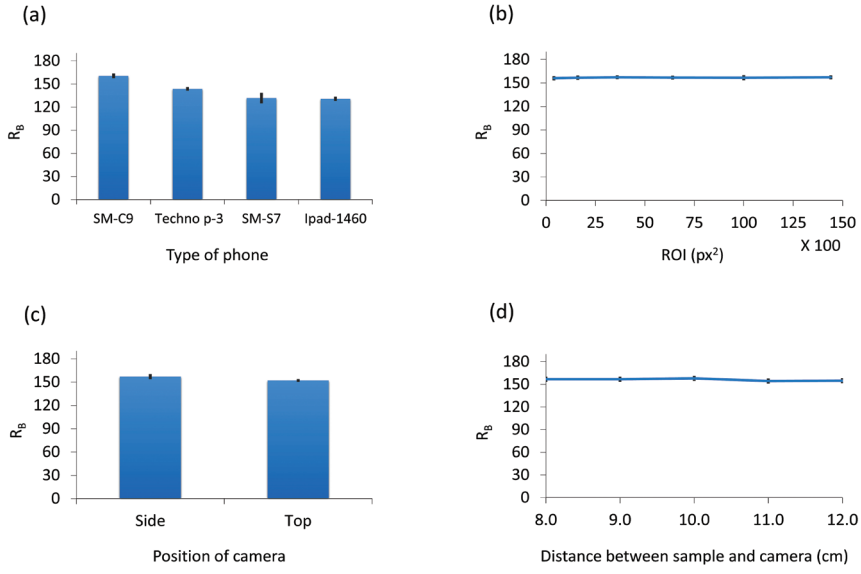

Figure 3. Optimization of smartphone digital image colorimetry parameters: (a) Type of phone; (b) area of region of interest; (c) position of camera; (d) and distance between sample and camera. Optimum complexation conditions: Sample pH 5.5; concentration of ligand, 0.28 mmol L^{-1}; volume of sample solution, 3.0 mL; and complexation time, 1.0 min; R_B= 255-I_B, where, R_B and I_B are the response and intensity of the analyte solution in the B channel, respectively

samples. Expectedly, the response decreased with an increase in the volume of the sample solution due to dilution of the metal complex in the final solution (Figure 4c). As a result, the optimum sample volume was selected as 3.0 mL. The time interval between adding the ligand to the sample solution and detecting the yellow complex was defined as the complexation time, which corresponded to the vortex mixing period. The effect of complexation time was studied over the range of 1.0-5.0 min. The addition of the ligand resulted in instantaneous formation of a stable complex, as indicated by its consistent yellow (Figure 4d). Therefore, an optimum complexation time was selected as 1.0 min to ensure good repeatability.

Analytical performance

An aqueous calibration curve was plotted using standard aluminum solutions at concentrations ranging from 1.7 to 25.0 µmol L^{-1} to assess the analytical performance of the proposed method. A linear calibration graph was obtained with a coefficient of determination (R^2) of 0.9981 (Table 1). Repeatability was evaluated in terms of intraday and interday precision. The results, expressed as a percentage relative standard deviation were less than 2.2% and 3.1%, respectively. The limit of detection (LOD), calculated based on $3S_b/m$, where S_b is the standard deviation of the intercept and m is the slope of the regression equation, was found as 0.5 µmol L^{-1} (0.2 µg g^{-1}) and the limit of quantitation (LOQ), based on 10 S_b/m, was found to be 1.7 µmol L^{-1} (0.7 µg g^{-1}).

Determination of aluminum in antiperspirant products

All five of the analyzed samples were found to contain aluminum at concentrations in the range of 2.4 to 3.5% (w/w) (Table 2).

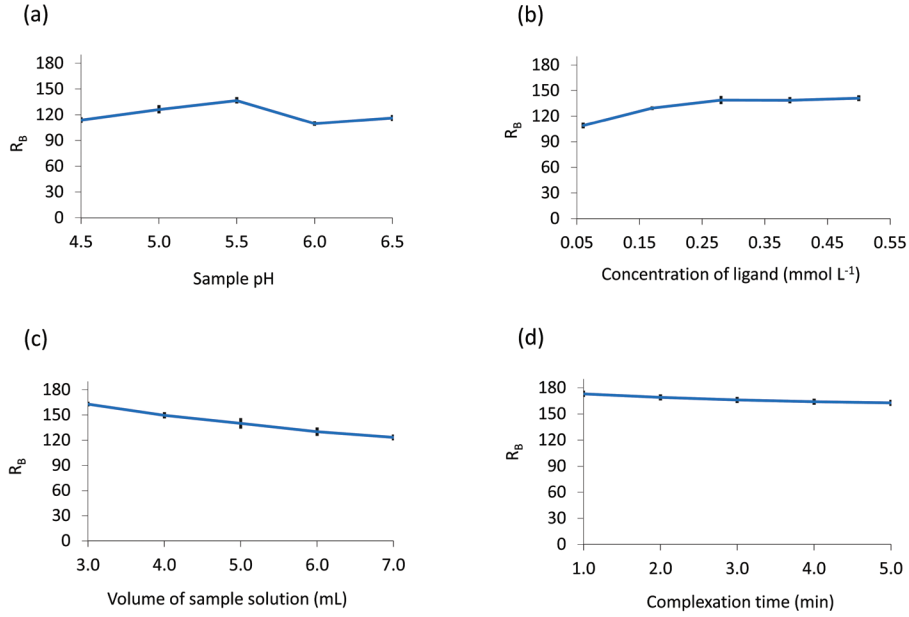

Figure 4. Optimization of complexation parameters: (a) Sample pH; (b) concentration of ligand; (c) volume of sample solution; (d) complexation time. Optimum smartphone digital image colorimetry conditions: Type of phone, SM-C9; area of region of interest, 6400 px^2; position of camera, side; distance between sample and camera, 10.0 cm; R_B= 255-I_B, where, R_B and I_B are the response and intensity of the analyte solution in the B channel, respectively

Table 1. Comparison of the analytical figures of merit of the proposed smartphone-digital image colorimetry method with high-performance liquid chromatography-diode-array detection for the determination of aluminum in antiperspirant products

Analytical technique	Regression equation[a]	R^{2b}	LOD[c]	LOQ[d]	LDR[e]	%RSD[f]	
						Intraday	Intraday
HPLC-DAD	*Y*= 1.38 (± 0.02)x + 0.93 (± 0.45)	0.9993	1.0 (0.3 µg g^{-1})	3.3 (1.1 µg g^{-1})	3.3-60.0	2.3	3.0
SDIC	*Y*= 2.36 (± 0.03)x - 0.0016 (± 0.40)	0.9981	0.5 (0.2 µg g^{-1})	1.7 (0.7 µg g^{-1})	1.7-25.0	2.2	3.1

[a]Response= slope (± SD) x [aluminum concentration (µmol L^{-1}) + intercept (± SD)
[b]Coefficient of determination
[c]Limit of detection (µmol L^{-1})
[d]Limit of quantitation (µmol L^{-1})
[e]Linear dynamic range (µmol L^{-1})
[f]Percentage relative standard deviation, n: 3
HPLC: High-performance liquid chromatography, DAD: Diode-array detector, SDIC: Smartphone digital image colorimetry, LOD: Limit of detection, LOQ: Limit of quantitation, RSD: Relative standard deviation, SD: Standard deviation

Table 2. Percentage recoveries of aluminum from antiperspirant products using smartphone digital image colorimetry and high-performance liquid chromatography-diode-array detection obtained with standard-addition and recovery tests

Sample[a]	Added (%, w/w)	Found (SDIC) (%, w/w)	%R[b]	Found (HPLC-DAD) (%, w/w)
APP1	-	2.9 (± 0.2)	-	3.2 (± 0.2)
	1.0	4.0	105.0	
	3.0	5.8	95.0	
	5.0	7.5	90.2	
APP2	-	2.4 (± 0.9)	-	3.3 (± 0.2)
	1.0	3.2	80.0	
	3.0	5.6	109.7	
	5.0	7.2	97.2	
APP3	-	2.7 (± 0.4)	-	3.2 (± 0.2)
	1.0	3.6	85.0	
	3.0	5.7	99.0	
	5.0	8.1	107.2	
APP4	-	2.6 (± 0.3)	-	2.4 (± 0.1)
	1.0	3.5	89.0	
	3.0	5.8	105.0	
	5.0	8.0	107.8	
APP5	-	3.3 (± 0.2)	-	3.5 (± 0.1)
	1.0	4.3	100.0	
	3.0	6.2	96.0	
	5.0	7.8	90.2	

[a]APP: Antiperspirant product, [b]Percentage recovery, a value obtained considering extraction yields from aqueous calibration graph, SDIC: Smartphone digital image colorimetry, HPLC: High-performance liquid chromatography, DAD: Diode-array detector

The accuracy of SDIC was checked using an addition-recovery test by spiking the samples with aluminum (as aluminum nitrate nonahydrate to the genuine samples) at three concentration levels (*i.e.*, 1.0, 3.0, and 5.0% w/w). The percentage absolute recoveries (%R), calculated from the aqueous calibration curve, were found to range between 80.0 and 109.7 (Table 2). An independent HPLC-DAD study found no statistically significant difference between the results obtained with both techniques (p>0.05) for all samples (Table 2), indicating good accuracy of the proposed SDIC method.

Comparison with other methods

The proposed SDIC method was compared with the HPLC-DAD method in terms of linearity, sensitivity, linear dynamic range, precision, analysis time and organic solvent consumption (Tables 1, 3). The main advantages of SDIC over HPLC-DAD are the significantly lower cost of purchase, operation and maintenance as well as the shorter analysis time, *i.e.*, 1 *vs.* 8 min, respectively (Table 3). Similarly, the proposed method was compared to other methods used for the determination of aluminum in personal care products. The results, summarized in Table 3, reveal that the proposed method is superior to the others in terms of cost, degree of greenness, and ease of implementation. Furthermore, it outperformed the others due to its zero organic solvent consumption, one-minute analysis time, and low energy dependence. Although paper platform for colorimetric determination-diffusive reflectance spectroscopy did not require any organic solvents, the analysis time was rather long.[12] The analysis time with flow-through potentiometric sensors was only 0.5 min, but it required 3 mL of organic solvents *per* sample.[10] SDIC was more sensitive than the other methods except for dispersive liquid-liquid microextraction-UV/Vis[27] but the analysis time was longer.

CONCLUSION

In this study, SDIC was developed for the determination of aluminum in APPs. Quercetin was used as a ligand to form an easily detectable yellow complex with aluminum. The proposed method could be used to accurately determine aluminum in genuine samples, as demonstrated by HPLC-DAD and addition-recovery tests. In comparison to the latter method and others used in the literature for determining aluminum in personal care products including antiperspirants, the proposed method

Table 3. Comparison of smartphone digital image colorimetry with other methods for the determination of aluminum in different types of samples

Method[a]	Sample	Analysis time (min)	$V_{org.}$[b] (mL)	LOD[c] (µg mL^{-1})	R^2[d]	RSD[e]%	References
CSPE-DRS	Cookware, antacids, hygienic care products	67	159	0.18	0.992	8.8	[11]
LLME-HPLC-UV/Vis	Antiperspirants	46	12.5	1.24	0.997	5.5	[8]
FI-UV/Vis	Antiperspirants	44	60	0.0161	0.9992	<2	[9]
DLLME UV/Vis	Water, Food, biological, pharmaceuticals	32	0.2	0.0002	0.9975	1.8-3.3	[27]
PPC-DRS	Antiperspirants	12	0	3.06	0.999	<5.0	[12]
FTPS	Deodorants	0.5	3	0.5	0.994	3	[10]
HPLC-DAD	Antiperspirants	8	0	0.027	0.9993	3.0	This study
SDIC		1	0	0.013	0.9981	3.1	

[a]CSPE-DRS: Coupled colorimetric solid-phase extraction-diffusive reflectance spectroscopy, LLME-HPLC: Liquid-liquid extraction-high-performance liquid chromatography, FI-UV/Vis: Flow injection-ultraviolet/visible spectrophotometry, DLLME: Dispersive liquid-liquid microextraction, PPC: Paper platform for colorimetric determination, FTPS: Flow-through potentiometric sensors, DAD: Diode-array detector, SDIC: Smartphone digital image colorimetry, [b]Volume of organic solvents consumed, [c]Limit of detection, [d]Coefficient of determination, [e]Percentage relative standard deviation, RSD: Relative standard deviation, LOD: Limit of detection

offers several significant advantages, including low operation and maintenance costs, short analysis time, high degree of greenness, simplicity of execution, zero organic solvent consumption, minimal energy dependency, portability, and high potential for on-field analysis. The obtained results show that SDIC is a viable alternative to sophisticated instrumental techniques that are not easily owned and/or operated by every laboratory for the determination of inorganic and organic analytes in various samples.

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Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.A., U.A., Design: S.A., U.A., Data Collection or Processing: S.A., U.A., J.C., Analysis or Interpretation: S.A., U.A., J.C., Literature Search: S.A., U.A., Writing: S.A., U.A., J.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Advantages and Disadvantages of Two *In Vitro* Assays in Evaluating Aromatase Activity: "A Cell-Based and a Cell-Free Assay"

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ABSTRACT

Objectives: Aromatase is an enzyme that catalyzes the conversion of androgens to estrogens. While inhibition of aromatase is a useful approach for treating breast cancer, it may also have toxicological consequences due to its endocrine disrupting/modulating effect. In this study, sensitivity and performance of two *in vitro* assays -a cell free and a cell-based- for evaluating aromatase activity were investigated by testing known aromatase inhibitors and partial validation of the methods was performed. Advantages and disadvantages of these methods are also discussed. **Materials and Methods:** Aromatase activity was evaluated *via* two *in vitro* models; direct measurement with a cell-free assay using a fluorescent substrate and recombinant human enzyme and indirect evaluation with a cell-based assay where cell proliferation was determined in estrogen receptor positive human breast cancer cells (MCF-7 BUS) in the absence of estrogen and the presence of testosterone. **Results:** In the cell-free direct measurement assay, reference compounds ketoconazole and aminoglutethimide have been shown to inhibit the aromatase enzyme with half-maximal inhibitory concentration (IC_{50}) values concordant with literature. In cell-based indirect measurement assay, only ketoconazole dose-dependently inhibited cell proliferation with 3.47 x 10^{-7} M IC_{50}. Inter-assay and intra-assay reproducibility of both methods was found to be within acceptable deviation levels. **Conclusion:** Both methods can be successfully applied. However, to evaluate the potential aromatase activity of the novel compounds *in vitro*, it seems better to perform both the cell-based and the cell-free assays that allows low-moderate biotransformation and eliminate cytotoxicity potential, respectively.

Key words: Aromatase inhibition, cell-based assay, cell-free assay, *in vitro*

INTRODUCTION

Endocrine disruptors are exogenous compounds, which cause adverse effects by altering endocrine system functions.[1] These compounds have several mechanisms of action, one is to modulate the cytochrome P450 (CYP450) enzymes involved in steroid hormone synthesis/metabolism.[2]

Aromatase is a member of CYP450 enzyme superfamily, which catalyzes the conversion of androgens to estrogens during the last step of steroidogenesis.[3] This conversion by aromatase is a rate-limiting step in estrogen synthesis and the enzyme is responsible for maintaining a homeostatic balance between androgens and estrogens. Aromatase is involved in numerous physiological functions such as reproduction, development, behavior as well as pathologies such as hormone-dependent cancers. Especially in postmenopausal women, local estrogen synthesis *via* aromatization of androgens plays a crucial role in the development of estrogen-dependent breast cancer.[3] Therefore, inhibition of aromatase is a useful approach for treating hormone-dependent breast cancer. On the other side, inhibition of this enzyme may have toxicological consequences because of endocrine disruption/modulation.

In this paper, aromatase activity was measured by two *in vitro* assays. The first one is a high throughput screening assay,

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where a fluorescent substrate and recombinant human enzyme are used, and the enzyme activity is detected directly *via* use of the substrate. In the second assay, enzyme activity is indirectly evaluated *via* proliferation of the estrogen receptor-positive human breast cancer cells, MCF-7 BUS, in the presence of testosterone in an estrogen-free medium. The sensitivity and performance of both assays were evaluated by testing known aromatase inhibitors and partial validation of the methods was performed. Advantages and disadvantages of cell-based and cell-free assays are discussed.

MATERIALS AND METHODS

MCF-7 BUS cells were kindly provided by Prof. Ana Soto from Tufts Institute and maintained at 37°C in 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Reference compounds (ketoconazole and aminoglutethimide) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific. An aromatase activity assay kit was purchased from Corning Incorporated (New York, USA).

Direct measurement of aromatase activity

Direct measurement of aromatase activity was evaluated by CYP19A/7-methoxy-4-trifluoromethyl coumarin (MFC) screening kit from Corning Incorporated (New York USA). Reaction substrate MFC is converted to 7-hydroxytrifluoromethyl coumarin by aromatase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) generating system. Thus, reduction of in fluorescence intensity refers to aromatase inhibitor activity.[4,5] Enzyme reactions were performed, according to the manufacturer's protocol as indicated in detailed previously.[6] IC_{50} values of reference materials were obtained using GraphPad Prism5 software.

Intra-assay reproducibility was determined *via* calculation of the mean and standard deviation (SD) values of the enzyme activity, which were measured in 5 different wells on the same day, while interassay reproducibility was determined *via* calculation of the values from 3 different days.

Indirect measurement of aromatase activity

If the estrogen-dependent cells are seeded in estrogen-depleted media, cell proliferation occurs *via* aromatization of androgens. Thus, aromatase activity can be measured indirectly in MCF-7 cells by evaluating cell viability in the medium with testosterone/without estrogen, according to the method[7] with minor modifications as previously described.[6]

Briefly, MCF-7 BUS cells were plated in 96 well plates at a density of 6000 cells/well in DMEM supplemented with 10% FBS and incubated at 37°C in a humid atmosphere containing 5% CO_2. After 48 hours of attachment, the medium was replaced with DMEM without phenol red supplemented with 10% charcoal stripped FBS, 1% sodium pyruvate and 1% non-essential amino acid solution containing either testosterone (10 µM) alone or testosterone and the tested compounds together. A control group was also included, in which the cells were grown in estrogen-depleted media without any testosterone or test molecule. Following 5 day incubation period, cell viability was assessed *via* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The medium was removed, cells were washed with phosphate buffered saline and then incubated with MTT (1 mg/mL) for 4 h at 37°C. MTT solution was removed and formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was recorded at 550 nm on a microplate reader. The ratio of the absorbance of treated samples to the absorbance of control (taken as 100%) was expressed as percentage cell viability.

To evaluate performance and the sensitivity of the assay, cells were incubated with 17-β-estradiol (1 nM)-, and testosterone (1 and 10 µM) for 5 days in the presence and absence of aromatase inhibitors.

Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed using student's *t*-test. Differences were considered significant $p<0.05$. *P* values are given in figure legends.

RESULTS AND DISCUSSION

In this study, aromatase activity was measured using two different *in vitro* assays; a cell free, direct measurement assay and a cell-based, indirect measurement assay. Performance and sensitivity of the assays were compared using reference compounds, *i.e.* ketoconazole, general CYP inhibitor and a well-known aromatase inhibitor, *i.e.* aminoglutethimide.

In the cell-free aromatase activity assay, human recombinant aromatase enzyme (CYP19) and a fluorescence substrate MFC were used. In NADPH generating system, fluorescence intensity is reduced because of demethylation of MFCs by CYP19 and enzyme activity is calculated fluorometrically. Since this method is performed in 96 well plate format and allows high throughput screening, different groups have previously used it to evaluate novel aromatase inhibitors.[8]

Sensitivity and performance of the direct measurement assay in our laboratory conditions was evaluated by using a known aromatase inhibitor aminoglutethimide and a general CYP inhibitor ketoconazole. Ketoconazole and aminoglutethimide have been shown to inhibit aromatase in the direct measurement assay with 2.3×10^{-6} M and 4.7×10^{-7} M IC_{50} values, respectively (Table 1). Compared to the IC_{50} values from the literature, our results were found to be concordant with the literature (Table 1).[9]

Inter-assay and intra-assay reproducibility of the direct measurement assay was also evaluated by measuring enzyme activity in the presence of a fixed amount of recombinant enzyme and substrate (50 µM MFC). Intra-assay and inter-assay

Table 1. IC_{50} values of ketoconazole and aminoglutethimide that were obtained from literature and from direct aromatase activity assay in the present study[9]

Reference compounds	Literature IC_{50}	Detected IC_{50}
Ketoconazole	2.0×10^{-6} M	2.3×10^{-6} M
Aminoglutethimide	6.0×10^{-7} M	4.7×10^{-7} M

coefficients of variation values were 2.8% and 10%, respectively (Table 2). According to these results, direct measurement assay is found to be in an acceptable reproducibility range.

Indirect measurement assay is performed in MCF-7 BUS cells by evaluating proliferation of the cells in estrogen deprived but testosterone-added media. MCF-7 BUS is a well-established estrogen receptor-positive cell line and depends on estrogen for proliferation. Cells possess aromatase activity.[10] In this study, we also performed western blotting (data not shown) and confirmed the expression of aromatase in our cell line. Since cell proliferation depends on the presence of estrogens, in the absence of estrogen but in presence of testosterone, cell proliferation depends on the aromatization of testosterone to estrogen *via* aromatase enzyme,[7] that is the principle of this indirect measurement assay.

Performance and the sensitivity of the indirect measurement assay was evaluated by using reference compounds (estradiol and testosterone) (Figure 1) in the presence and or absence of aromatase inhibitors. As expected, 17-β-estradiol significantly increased cell proliferation (approximately 2 fold) comparing to the control group. Testosterone also increased cell proliferation in a dose-dependent manner. This effect was reduced by the aromatase inhibitors, *i.e.* aminoglutethimide (100 µM) and ketoconazole (5 µM), indicating that cell proliferation was estrogen-dependent and catalyzed by aromatase activity of the cells (Figure 1).

Table 2. Inter-assay and intra-assay reproducibility values of direct aromatase activity assay

	Intra-assay reproducibility	Inter-assay reproducibility
Mean of FI ± SD	2.33 ± 0.07 (n: 5)	2.27 ± 0.23 (n: 3)
% Coefficient of variation	2.8	10

FI: Fluorescence intensity, SD: Standard deviation

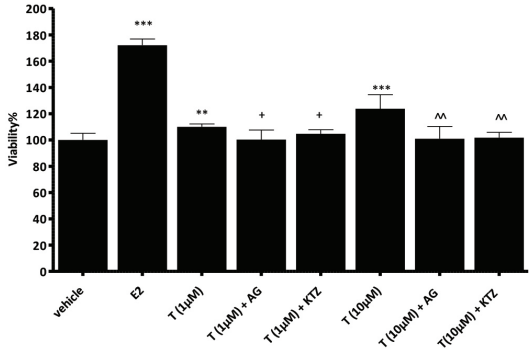

Figure 1. Effect of testosterone, aminoglutethimide (100 µM), and ketoconazole (5 µM) on MCF-7 BUS cell proliferation. Bars show percentage viability values compared to control group (mean ± SD). Statistical analysis was performed by using student's *t*-test.

***p*<0.005 *vs* vehicle, ****p*<0.001 *vs* vehicle, ^{+}p<0.05 *vs* T (1 µM); ^^*p*<0.005 *vs* T (10 µM)

SD: Standard deviation

After that, cells were incubated with 10 µM testosterone and varying concentrations of ketoconazole or aminoglutethimide for 5 days to obtain IC_{50} values in the indirect measurement assay. We found that ketoconazole (0.05-20 µM) inhibited cell proliferation because of aromatization of testosterone to estradiol (Figure 2) with 3.47 x 10^{-7} M IC_{50} value. On the other hand, aminoglutethimide did not inhibit cell proliferation in a dose dependent manner (data not shown). Therefore, IC_{50} value of aminoglutethimide could not be calculated.

Inter- and intra-assay reproducibility of the indirect measurement assay was evaluated. Intra-assay reproducibility was calculated using the results of the estradiol and testosterone obtained from four different wells on the same day. % coefficient variation values of testosterone and estradiol were 2.7% and 7.4%, respectively (Table 3). For the inter-assay reproducibility, percent coefficient variation values of testosterone and estradiol were calculated as 2.5% and 2.6%, respectively, which were obtained from the results of the experiments conducted over four different days. According to these results, it was concluded that the indirect measurement assay has a high rate of successful replications and works successfully.

While the IC_{50} value of ketoconazole was found to be 2.5 µM in the direct activity measurement method, it was about 10

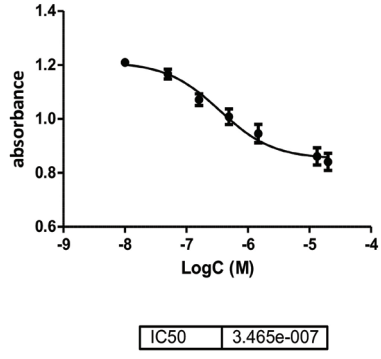

Figure 2. Inhibitory effect of ketoconazole on indirect aromatase activity. Cells were incubated with testosterone (10 µM) and ketoconazole for 5 days

Table 3. Inter-assay and intra-assay reproducibility values for indirect aromatase activity measurement assay

	Intra-assay reproducibility (n: 4)		Inter-assay reproducibility (n: 4)	
	Mean of % control ± SD	% coefficient of variation	Mean of % control ± SD	% coefficient of variation
Testosterone (10^{-5} M)	134 ± 4	2.7	134 ± 3	2.5
Estradiol (10^{-9} M)	202 ± 15	7.4	199 ± 5	2.6

SD: Standard deviation

times lower (0.35 μM) in the indirect aromatase activity. This difference is thought to be because of possible metabolites of ketoconazole. Nevertheless, in a study conducted in a primary culture system of rat hepatocytes, major metabolite of ketoconazole (*N*-deacetylated ketoconazole) has a more potent cytotoxic effect in an MTT assay.[11] Therefore, the reason for this difference may be potential cytotoxic, estrogen receptor antagonists or of aromatase expression modulator effects of the possible metabolites. It was also demonstrated by Yan et al.[12] that ketoconazole downregulates aromatase gene expression in goldfish. So, lower IC_{50} value of ketoconazole in cell-based indirect measurement assay may be the consequence of both inhibition of aromatase enzyme and downregulation of aromatase expression.

It should also be kept in mind that substance concentration interacting with the active site of aromatase enzyme cannot be the same in cell-based and cell-free assay. There are lots of biological steps in cell-based assays, such as passaging through the membranes, entering the cells and metabolism, which can affect the results.

However, the cell-based method has a metabolic capacity compared to the direct measurement assay. Therefore, it is possible to evaluate the potential effects of the active metabolites, which makes it a more advantageous method in reflecting the physiological state in a more realistic way.

CONCLUSION

In conclusion, partial validation results of this study indicate that direct and indirect measurement assays can be used for evaluating aromatase activity, but both of them have some advantages and disadvantages indeed. Therefore, it seems better to perform this cell-based and cell-free assays together to evaluate the potential aromatase activity of the novel compounds to comment on the results correctly. Additional tests like cytotoxicity, effect on enzyme expression levels should also be performed to prevent misinterpretation of the indirect measurement assay results.

Ethics

Ethics Committee Approval: Ethical approval is not required for the study.

Informed Consent: Not necessary.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: E.İ.E., H.G.O., Design: E.İ.E., S.Ö.S., H.G.O., Data Collection or Processing: E.İ.E., S.Ö.S., Analysis or Interpretation: E.İ.E., S.Ö.S., Literature Search: E.İ.E., S.Ö.S., Writing: E.İ.E., H.G.O.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Identification of Bioactive Compounds of the Endophytic Fungus *Aspergillus egypticus*-HT166S Inhibiting the Activity of Pancreatic α-Amylase

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ABSTRACT

Objectives: *Diabetes mellitus* (DM) is a worldwide increasing problem, associated with development of hyperlipidemia, coronary heart disease, hypertension, and other chronic diseases. Decreasing of glucose absorption by inhibition of α-amylase is one of the therapeutic approaches to retard diabetes type 2. Pancreatic α-amylase (PA) inhibition widely studied mechanism for determination of potential of natural compounds as antidiabetic agents. The aim of this work was identification of inhibitory secondary metabolites produced by *Aspergillus egypticus*, isolated from *Helianthus tuberosus*.

Materials and Methods: The PA inhibitory activity of the secondary metabolites determined using iodometric method. Isolation of inhibitory compounds was carried out by column chromatography, thin layer chromatography and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Results: It was found that the inhibitory concentration of a compound, K-10 (R_f: 0.74), isolated from metanolic extract of *A. egypticus* was 4.82 mg/mL. LC-MS/MS analysis of K-10 showed polymethoxylated flavones (PMF).

Conclusion: The fungal endophyte *A. egypticus*-HT166S can be considered a source of PMF as potential agents for developing new PA inhibitors.

Key words: *Diabetes mellitus*, endophyte, diabetes, secondary metabolites, inhibitory activity, column chromatography, LC-MS/MS

INTRODUCTION

Diabetes mellitus (DM) is a metabolic syndrome characterized by hyperglycemia and abnormalities in the metabolism of carbohydrates, fats, and proteins, leading to insulin secretion or/and sensitivity.[1] The consumption of a high-carbohydrate diet causes postprandial hyperglycemia with the development of a complete symptomatic picture of type 2 DM.[2] The number of patients with diabetes is growing dramatically worldwide. According to World Health Organization forecasts, by 2040, the number of patients with diabetes will be 642 million. Simultaneously, 90% of the total number of patients are with type 2 DM.[3]

DM therapy is aimed to prevent hyperglycemia and subsequent complications associated with cardiovascular factors, and in general, to improve the quality of life.

One of the treatment approaches of type 2 DM is reducing postprandial blood glucose, caused by delayed glucose absorption by inhibition of polysaccharide breakdown to mono- and disaccharides by α-amylase and α-glucosidase in the intestine.[2,4-7] Inhibitors of these enzymes prolong the total carbohydrate digestion time, contributing to a decrease in the rate of glucose absorption, followed by blocking the postprandial increase in glucose levels.[8] However, most known to date inhibitors (acarbose, miglitol, and voglibose) have severe undesirable side effects; abdominal pain, bloating, diarrhea, kidney cancer, liver damage, and acute hepatitis.[6,9] The development of new natural inhibitors of pancreatic α-amylase and α-glucosidase that can restore normoglycemia without side effects requires appropriate research in herbal medicine and alternative medicine.

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Some secondary metabolites of the antidiabetic plants successfully demonstrate the properties of inhibitors of carbohydrate degrading enzymes, which may help control type 2 diabetes.[2,4,5,7] Recently, endophytes of medicinal plants are the most attractive source of natural product sources with high structural diversity and bioactivity and have several advantages over plant raw materials.[10] The endophytes of diabetic plants are of particular interest since they can probably produce compounds that mediate the antidiabetic properties of the host plants.[4-6]

For example; *Colletotrichum capsici* isolated from *Eugenia cumini* L. has strong antibacterial efficacy and antidiabetic action and contains fatty acids and phenolic compounds.[11] Similar results were reported by Govindappa et al.[12] who *in vitro* determined the antidiabetic, antioxidant, and anticholinesterase activities of the methanolic extract of the endophyte *Cladosporium uredinicola* isolated from endemic plant *Calophyllum tomentosum* Wight. Phytochemical analysis of the fungal extract showed presence of flavonoids, tannins, alkaloids, glycosides, phenols, terpenoids, and coumarins.[12]

In our previous studies of the roots, stems, leaves, and tubers of *Helianthus tuberosus* L. growing in Uzbekistan, there were obtained 17 endophytic fungal isolates related to different genera.[13] The most active *Aspergillus egypticus*-HT166S inhibited α-amylase activity for more than 80%.[14]

The fractionation of crude ethylacetate extract of *A. egypticus*-HT166S metabolites by the stepwise extraction with polar and non-polar solvents, it was found that the metabolites with the highest inhibitory activity were recovered in the methanol fraction.[15]

In this regard, this work aims to separate and study of inhibitory compounds in the methanol extract of the endophytic fungus *A. egypticus*-HT166S.

MATERIALS AND METHODS

Cultivation of A. egypticus-HT166S endophytes

The endophytic fungus *A. egypticus*-HT166S, previously isolated from the stem of *H. tuberosus*, was grown submergely in Czapek-Dox medium on an orbital shaker at 160 rpm for 7 days.[14] The biomass was separated from the culture liquid by centrifugation at 6000 rpm.

Fractionation of secondary metabolites

Fractionation of secondary metabolites of *A. egypticus*-HT166S biomass was carried out according to the scheme proposed by Kumar et al.[5], including sequential extraction with water, methanol: hexane (1:1), and butanol. As a result, a methanol extract was obtained with an inhibitory activity of 75.4%. The extract was dried on a rotary evaporator and 1 mL of dimethyl sulfoxide was added. The resulting dry methanol extract was stored at 4°C for reuse.[15]

Column chromatography

The methanol extract (500 mg) was applied to a column (2 x 25 cm) filled with 20 g of silica gel (100/250, LaChema) and eluted in chloroform: methanol 50:1 ~ 1:1 graduated solvent system to yield fractions at a flow rate of a mobile phase 1.5 mL/min. Those fractions with the same R_f value after thin layer chromatographic analysis were pooled together and evaporated till the dried fraction (A1-M12) was obtained.[11]

Thin layer chromatography

Samples of 25 µL were loaded onto plates (Sigma-Aldrich, Germany) and chromatographed in the chloroform: Methanol (5:1) system. The plates were scanned with ultraviolet light at a wavelength of 254 nm. Samples with the same R_f values were pooled and dried.

Liquid chromatography-tandem mass spectrometry (LC-MS) analysis

The mass spectra of the fractions obtained on a Q-TOF LC-MS Agilent Technologies 6520V device under the following conditions: ESI positive ion mode, positive ion electrospray method, drying gas flow rate of 5 L/min, drying gas temperature of 300°C, ion acceleration voltage on the skimmer 35 V, fragmented 175 V, range MS 150-1000 m/z, target MS-MS 50-1000 m/z, collision energy - 30, 40, 50, 65. Samples injected onto a Zorbax SB C18 (3 µm, 150 x 0.5 mm) column (Agilent Technologies 1200) with a mobile phase: A) 0.1% formic acid, B) acetonitrile + 0.1% formic acid. Elution on the Agilent Technologies 1260 Cap pump at 15 µL/min: 5 min 60%, 15-20 min - 90%, 25 min - 60% of the mobile phase 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.[16] The starch solution prepared as a substrate in an amount of 1 g/10 mL of water, boiled for 2 min, the sample volume was adjusted to 100 mL with distilled water. 100 mL of pancreatic α-amylase (0.1 M Na-acetate buffer is 13 mL at pH 7.2), 100 µg of endophyte extract, 2 mL of acetate buffer were incubated for 10 min at 30°C for 2 mL 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: $(A_0-A_t)/A_0 \times 100\%$, where A_0 is the absorption of the control sample, and A_t is the absorption of the experimental sample, respectively. As a comparison drug, acarbose was used from a commercial drug "Glucobay" (Bayer Pharma AG, Germany) was used.

The concentration causing 50% inhibition of pancreatic α-amylase (IC_{50}) by the test samples was quantified as described by Murado et al.[17]

Calculation of results

The values are expressed as the mean value of ± standard deviation (n: 3). Statistical analysis has not been performed for evaluation of the results.

RESULTS AND DISCUSSION

As mentioned above, for the isolation of bioactive substances with high inhibitory activity, the total ethyl acetate extract biomass of *A. egypticus*-HT166S was fractionated in solvents of different polarities and the highest inhibitory activity was extracted by methanol.[15]

Figure 1 demonstrates the total ion chromatogram of the initial methanol fraction of *A. egypticus*-HT166S. As can be seen from the chromatographic data, the methanol fraction contained many substances, three of which are represented by relatively high peaks.

As can be seen from the data in Table 1, the inhibitory activity of the obtained metabolite samples varies widely from 7.0 to 76.2%. Simultaneously, the highest level of inhibitory activity was noted in the K-10 fraction with an R_f value of 0.74 and the content of secondary metabolites constituting 10% of the initial weight of the dry methanol fraction (Table 1).

Qualitative phytochemical analysis of K-10 fraction showed a positive reaction to flavonoids, as evidenced by the formation of an intense yellow by 20% NaOH and disappearance of color by 70% HCl.[18]

Note that over the past 20 years, scientific attention has been paid to natural compounds, such as flavonoids, which serve as antidiabetic agents. Flavonoids improve the pathogenesis of diabetes and its complications by regulating glucose metabolism, liver enzyme activity, and lipid profile. *In vitro* and

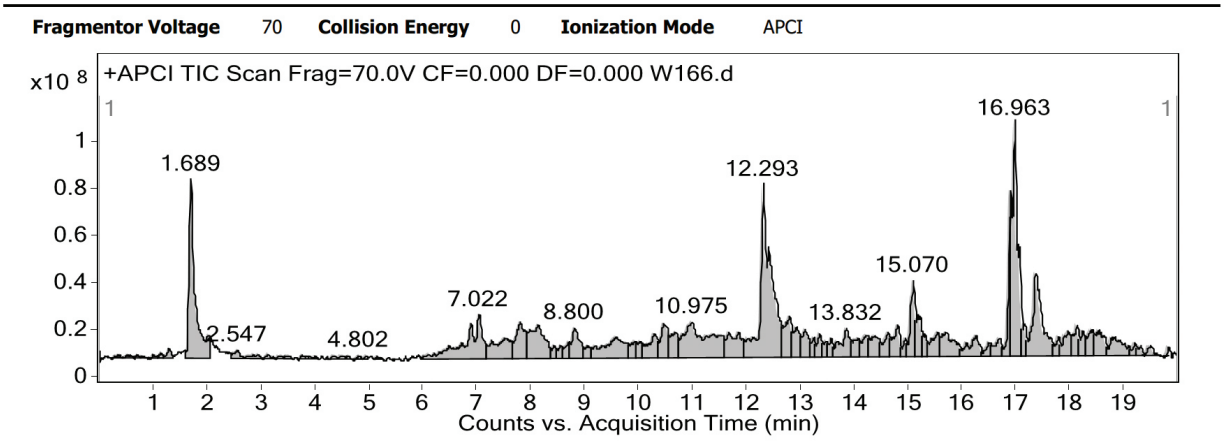

Figure 1. Total ion chromatogram of the total methanol fraction of the biomass *Aspergillus egypticus*-HT166S.

Because of fractionation of the methanol extract by column chromatography in a gradient concentration of chloroform: methanol 50:1 ~ 1:1, twelve fractions (A1-M12) were obtained, which were dried on a rotary evaporator. Each obtained fraction of metabolites was further evaluated by inhibition of pancreatic amylase

Table 1. Content and inhibitory activities of samples obtained from purification on a column of the total methanol fraction of *Aspergillus egypticus*-HT166S

Fractions	R_f	Dry weight, %	α-Amylase inhibition, %
A-1	0.13	3.8 ± 0.02	14.6 ± 0.29
B-2	0.22	4.5 ± 0.03	15.2 ± 0.29
C-3	0.30	7.8 ± 0.37	28.6 ± 0.30
D-4	0.37	4.1 ± 0.35	17.7 ± 0.27
E -5	0.44	3.2 ± 0.33	25 ± 0.28
F-6	0.48	2.7 ± 0.13	15 ± 0.34
G-7	0.52	3.1 ± 0.04	24.3 ± 0.29
H-8	0.54	6.5 ± 0.34	7.0 ± 0.26
J-9	0.6	5.2 ± 0.24	24 ± 0.30
K-10	0.74	10 ± 0.17	76.2 ± 0.29
L-11	0.86	6.6 ± 0.14	18.8 ± 0.30
M-12	0.97	2.3 ± 0.03	-
Total methanol fraction	-	100	75.4 ± 0.27

Each value is the average of three analyses ± standard deviation

in vivo studies have shown that they can prevent diabetes and its complications.[19] In identifying flavonoids, we referred to the experimental data of Zhang et al.[20], who developed a fast and efficient analytical method of tandem mass spectrometry with high performance liquid chromatography for the structural characterization of flavonoids from complex extracts of traditional Chinese medicines.

The mass spectral analysis of the bioactive K-10 sample showed compounds with molecular ions $[M + H]^+$ with *m/z* 359.0, *m/z* 345.0, and *m/z* 327.0 (Figure 2).

On comparative analysis of our results with the literature data, the compounds were assigned as polymethoxylated flavones (PMF).

PMF is a subclass of flavonoids in which all or almost all hydroxyls are blocked by methylation, have high oral bioavailability, exhibit anti-allergic, antioxidant, antibacterial, antiproliferative, anti-inflammatory, and anti-cancer activities.[21] The literature provides information on PMFs, mainly nobiletin, tangeretin, sinensetin, and isosinensetin from citrus plants, and discusses their antidiabetic effects *in vitro*.[22] For example; nobiletin, the polymethoxylated flavonoid, reduces the inflammation associated with gestational DM (GDM), a condition in which pregnant women suffer from carbohydrate intolerance during pregnancy. Nobiletin improved glucose metabolism in animal and human GDM models and may be a novel therapeutic agent for preventing GDM.[23] Sundaram et al.[24] evaluated the antihyperglycemic potential of PMF tangeretin on the activity of key enzymes of carbohydrate and glycogenic metabolism in control rats and rats with streptozotocin-induced diabetes. Studies have revealed that tangeretin modulates the activity of liver enzymes due to increased insulin secretion and reduces blood glucose levels in rats with streptozotocin-induced diabetes due to its antioxidant potential.[24]

Comparative analysis of the inhibitory activities of the purified sample K-10 and acarbose as a reference standard showed almost the same low IC_{50} values of 4.82 mg/mL and 4.74 mg/mL, respectively, compared to IC_{50} of the total methanol extract (5.53 mg/mL).

The results obtained indicate that the inhibitory activity of the purified fraction, K-10, is comparable to that of the reference drug (acarbose), and indeed contains bioactive compounds with potential inhibitory activity against α-amylase (Figure 3).

CONCLUSION

Natural bioactive compounds can inhibit α-amylase, which are the best and most useful substances to lower the blood

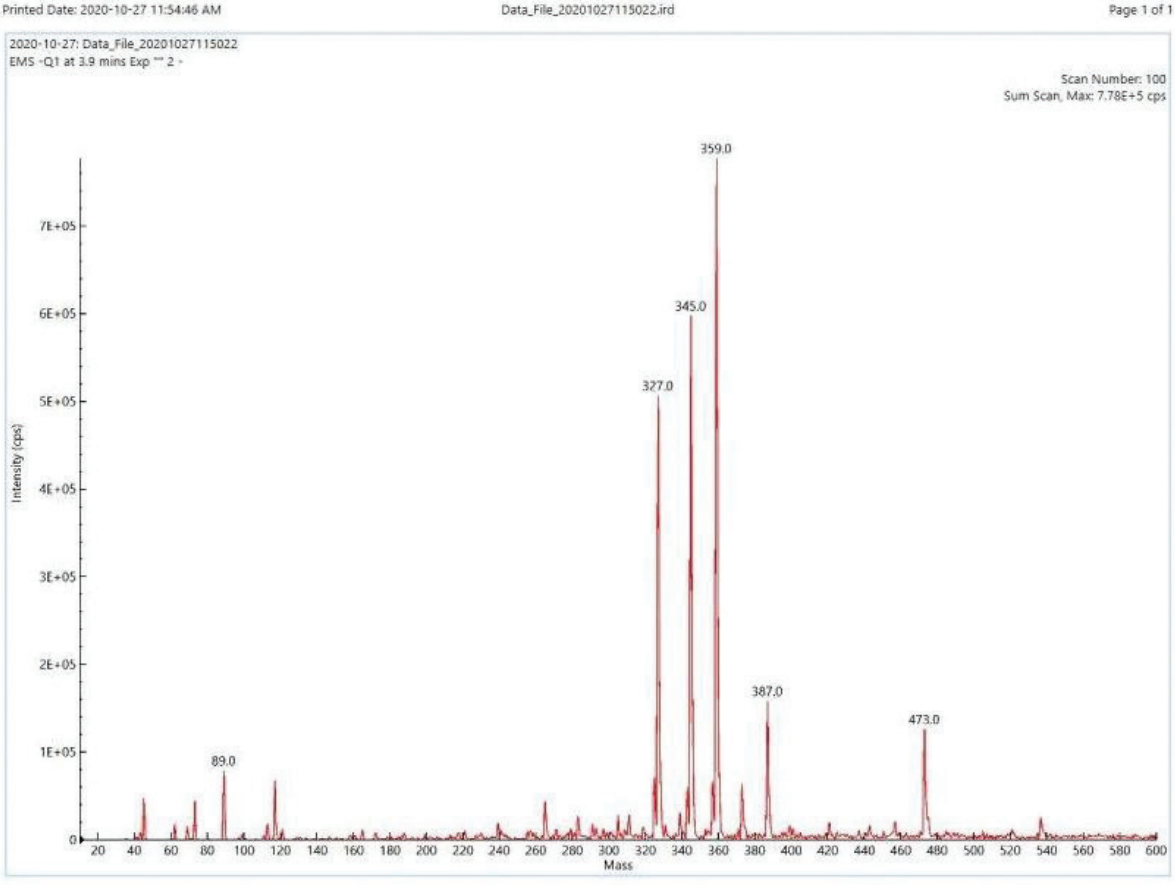

Figure 2. Mass spectral analysis of the fraction K-10

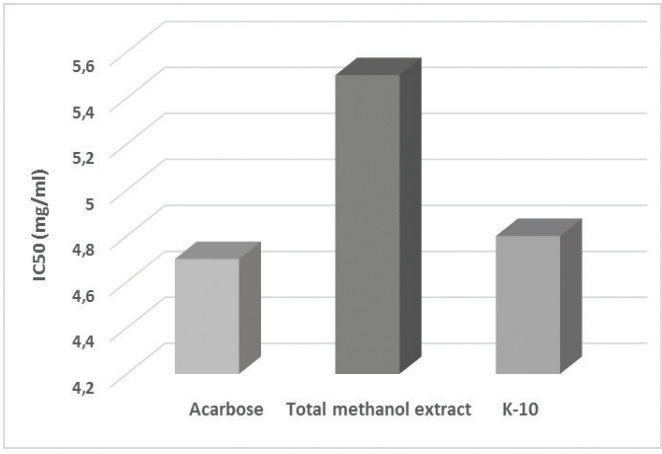

Figure 3. IC_{50} (in µg/mL) of total methanol extract and K-10 fraction compared with acarbose

sugar. Inhibition of α-amylase is a successful manner in the prevention and therapy of diabetes. Therefore, the search for new sources of bioactive compounds, in particular, endophytic fungi, is an alternative way for developing new technologies for the production of microbial amylase inhibitors.

The presented studies show that the *A. egypticus*-HT166S endophyte from *H. tuberosus* produces PMF with high inhibitory activity against pancreatic α-amylase, comparable to the activity of the commercial drug, acarbose. However, to establish the structure of inhibitory PMF, it is necessary to conduct further studies using analysis - infrared and nuclear magnetic resonance spectroscopy.

Based on the data obtained, it can be concluded that the endophytic fungus *A. egypticus*-HT166S can be considered a new source of pancreatic amylase inhibitors for developing hypoglycemic drugs.

Ethics

Ethics Committee Approval: Not necessary.

Informed Consent: Not necessary.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: I.M., G.R., Concept: S.N., Design: S.N., Data Collection or Processing: D.R., Analysis or Interpretation: T.G., Literature Search: D.R., Writing: D.R.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

Evaluation of *Lonicera etrusca* var. *etrusca* Santi (Caprifoliaceae) Stem and Leaf in Terms of Anatomical Structures and Some Phenolic Compounds

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ABSTRACT

Objectives: The genus *Lonicera* includes medicinally important plants. Two varieties of *L. etrusca* have been recorded in Türkiye. Anatomical structures and phytochemical contents are important in the diagnosis and identification of medicinal plants. This study included stem and leaf anatomy of *L. etrusca* var. *etrusca* and high performance liquid chromatography (HPLC) analysis of the methanol extracts obtained from these parts.

Materials and Methods: Plant materials were collected from Ankara. Methanol extracts were prepared from the stems and leaves by ultrasonic bath. The amounts of chlorogenic acid and caffeic acid that are major compounds in the stem and leaves, were determined by HPLC. For anatomical studies, specimens were preserved in 70% alcohol. Transverse and surface sections were prepared by hand. Detection of tissues was performed using Sartur reagent. Anatomical specimens were examined using a light microscope and microphotographed.

Results: In HPLC analysis, the highest amount of chlorogenic acid was determined in the leaf (1.148%), and the highest amount of caffeic acid (0.156%) was determined in the stem. In the anatomical analysis, it was observed that the stem was disc-shaped and hollow; pericycle is in a ring form, consists of fibre-like cells with thick walls and wide lumina; cork occurs adjoining pericyclic fibers; thin-walled pith cells containing dense druse crystals. The leaf lamina is bifacial in the transverse section; palisade and spongy parenchyma, both contain abundant starch grains; solitary druse crystals are sparse in the leaf mesophyll; the stomata were observed only on the lower surface with 3-5 subsidiary cells. With this study, *L. etrusca* var. *etrusca* has been clarified in terms of its anatomical structures and phenolic compounds.

Conclusion: The chemical contents and anatomical structures of the plant may contain important information that can be used in classification. This study may support in taxonomically classification for the *L. etrusca* var. *etrusca*.

Key words: *Lonicera etrusca* var. *etrusca*, Caprifoliaceae, HPLC, plant anatomy, Türkiye

INTRODUCTION

Caprifoliaceae family includes herbs and small trees, its native range is mainly north temperate and the medically important genera of the family are *Viburnum* L., *Lonicera* L., and *Sambucus* L.[1,2]

The genus *Lonicera* spreads from temperate and subtropical Northern Hemisphere to Malaysia and includes 200 accepted species and six species of *Lonicera* are naturally grown in Türkiye.[1-3] *L. etrusca* Santi grows naturally in Türkiye and is known for its yellowish corolla, red berries, obovate-oval leaves, and hollow young branches. Two varieties of *L. etrusca* are in Türkiye, *L. etrusca* var. *etrusca* Santi and *L. etrusca* var. *hispidula* Boiss., where *L. etrusca* var. *etrusca* is with the widest distribution in the genus *Lonicera* is grown in Türkiye. *L. etrusca* var. *etrusca* has young shoots, upper leaves, flowers glabrous,

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and lower leaves sparsely hairy to glabrous features, while *L. etrusca* var. *hispidula* are young shoots, upper leaves, flowers densely glandular-pubescent and lower leaves pubescent, eglandular.[3] Determination and identification are important for the plants as they are used in traditional medicine. The chemical contents and anatomical structures of the plant may contain important information that can be used in classification. Previous studies have been generally conducted on *L. etrusca*, regardless of varieties.

Lonicera is one of the most important genera in family, especially for its use in traditional Chinese medicine (TCM). *L. japonica* Thumb. is used for the treatment of febrile illnesses, sores, and swellings in TCM.[4-7] *L. caerulea* L. has therapeutic uses for hypertension, bacterial infections, and gastrointestinal disorders in northern Russia, China, and Japan.[8] *L. quinquelocularis* Hard. is used for its anti-inflammatory effect.[9] In Türkiye, *L. etrusca* var. *etrusca* is used for its diuretic effect,[10] while *L. caprifolium* L. is used as a laxative and emetic.[11]

The family contains valerianic acid, aucubin glycosides, saponins, coumarins, and cyanogenetic glycosides.[2] In phytochemical studies with *Lonicera* species, iridoids, bis-iridoids, triterpene saponins, phenolic acids, flavonoids, coumarins, anthocyanins, and monoterpene alkaloids were determined.[12-15] Fruits of *Lonicera* species (*L. altaica* Pall., *L. caerulea* L., and *L. edulis* Turcz. Ex Freyn) have major phenolic compounds such as flavonoids (*e.g.* rutin, quercetin, isoquercetin), anthocyanins (*e.g.* cyanidin, peonidin, delphidin glucosides), phenolic acids (*e.g.* gallic, vanilic, caffeic, ferrulic, chlorogenic, and genistic acids).[16] Phenolic compounds in plants have important biological and pharmacological effects. Studies have shown that these compounds have significant antioxidant, antimicrobial, anti-inflammatory, and anticancer effects.[17,18]

In this study, the leaf and stem anatomy of *L. etrusca* var. *etrusca*, which is widely distributed in Türkiye, was examined in detail and its characteristic structures were revealed. It was also evaluated in terms of phenolic compounds with significant biological activity. This is the first report on this species, in which both anatomical features are evaluated and phenolic compound quantification is performed.

MATERIALS AND METHODS

Plant material

The plant material was collected from Beynam Forest (Ankara, Türkiye) in 2020 (Figure 1). A voucher specimen was deposited in the Herbarium (AEF 30738) of Faculty of Pharmacy, Ankara University (Türkiye).

High performance liquid chromatography (HPLC) analysis

For HPLC analysis, methanol extracts of the air-dried stem and leaves were prepared. Powdered samples were extracted with methanol (Merck) using an ultrasonic bath. After the extracts were filtered and then concentrated with an evaporator.[19] HPLC analysis was performed on Agilent 1100 series with diode array detector using a Waters Spherisorb C18 column (25 cm x 4.6 mm, 5 µm) maintained at 40°C. In a gradient, 0.01% formic acid (Sigma-Aldrich) (A) and acetonitrile (Sigma-Aldrich) (B) were used as the mobile phase, with a flow rate of 1 mL/min. The detection wavelength was 300 nm and the injection volume was 20 µL.[20] After analysis, method validation was performed. Sample extracts (4 mg/mL) and stock standard solutions of each compound (500 µg/mL) were prepared by dissolving in methanol. For the calibration curve, chlorogenic acid (Sigma, Aldrich) and caffeic acid (Sigma, Aldrich) standards were investigated by injecting different concentrations, in triplicate. The precision of the method was determined by carrying on intra-day and inter-day variations and these variations were expressed by the relative standard deviation (RSD). Limit of detection (LOD) and limit of quantification (LOQ) were established at a signal/noise of 3 and 10, respectively. For LOD and LOQ, 10 injections of chlorogenic and caffeic acids were prepared and averaged. For recovery assay, it was carried out by spiking 3 different known concentrations of standards into the sample solution. These mixtures were analyzed by the same method used. Robustness of the method was evaluated by changing the mobile phase composition, column temperature, flow rate, and detector wavelength.

Figure 1. *Lonicera etrusca* var. etrusca

Light microscope analysis

The samples for anatomical studies were preserved in 70% alcohol. The transverse and surface sections were cut by hand with a razor blade in microscopic preparation form. Sartur solution was used in microscopic examinations.[21] The anatomical analysis and the microphotographs were taken using a Leica DM 4000B.

Statistical analysis

All analyses were conducted out at least in triplicate and the mean values were calculated. The mean, SD, and RSD data mentioned in the article, linear regression analysis and calculations were made using the Microsoft Excel 2016 program.

RESULTS

HPLC analysis

In this study, stem and leaves of *L. etrusca* var. *etrusca* were analyzed quantitatively for their chlorogenic and caffeic acid content using HPLC.

Yields of stem and leaf extracts were 8.59% and 13.69%, respectively. The chlorogenic and caffeic acid contents of the stem and leaves of *L. etrusca* var. *etrusca* extracts are shown in Table 1 and their HPLC chromatograms are given in Figures 2 and 3.

Table 1. Contents of chlorogenic acid and caffeic acid in stem and leaf methanol extracts (n: 3)

	Chlorogenic acid (% ± SD*)	Caffeic acid (% ± SD*)
Stem	1.043 ± 0.009	0.156 ± 0.0009
Leaf	1.148 ± 0.003	0.073 ± 0.001

*SD: Standard deviation

For calibration table, the ranges of 5 to 100 µg/mL the calibration plots for chlorogenic and caffeic acids were linear. The LOD values for chlorogenic and caffeic acids were determined as 0.093 µg/mL and 0.068 µg/mL; the LOQ values were determined 0.311 µg/mL and 0.229 µg/mL, respectively (Table 2).

Intra-day and inter-day variations were used to determine the precision. The results revealed that RSD values were always less than 2% (Table 3).

For a recovery assay, 3 different known concentrations of chlorogenic and caffeic acids were spiked into the sample solution. The mean extraction recoveries of chlorogenic and caffeic acids were in the range of 97.638%-99.795% and 97.260-102.092%, respectively (Table 4).

It was determined that some changes (mobile phase composition, column temperature, flow rate, and detector wavelength) made for the robustness of the test method did not have a significant effect on chromatographic resolution.

Stem anatomy

The transverse section of the stem is broadly grooved and hollow disc-like. The single-layer epidermis consists of square-

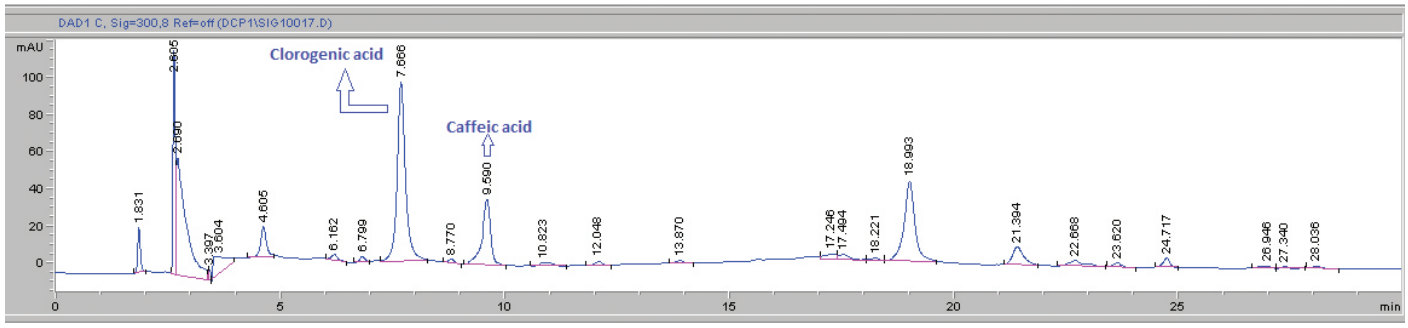

Figure 2. HPLC chromatograms of stem extract of *L. etrusca* var. *etrusca*
HPLC: High performance liquid chromatography

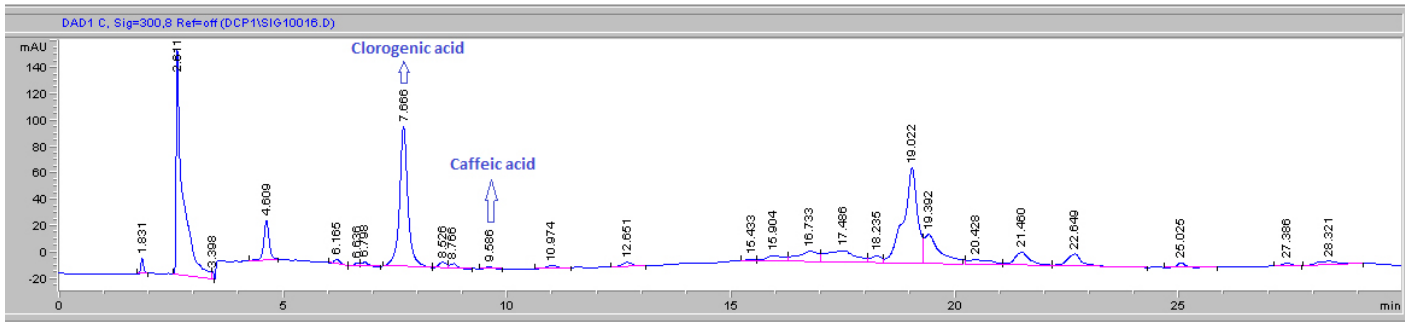

Figure 3. HPLC chromatograms of leaf extract of *L. etrusca* var. *etrusca*
HPLC: High performance liquid chromatography

Table 2. Calibration values for chlorogenic acid and caffeic acid

Standard	Calibration range (µg/mL)	Slope (a ± SD*)	Intersection (b ± SD*)	Correlation number (r^2 ± SD*)	LOD (µg/mL)	LOQ (µg/mL)
Chlorogenic acid	5-100	18.733 ± 1.547	6.692 ± 1.489	0.995 ± 0.003	0.093	0.311
Caffeic acid	5-100	81.837 ± 2.893	20.081 ± 4.751	0.996 ± 0.002	0.068	0.229

*SD: Standard deviation, LOD: Limit of detection, LOQ: Limit of quantification

Table 3. Intra-day and inter-day precision's data of the method

Standards	Amount (µg/mL)	Intra-day precision (RSD* %)	Inter-day precision (RSD* %)
Chlorogenic acid	5	1.694	1.425
	10	1.364	1.774
	25	0.516	0.256
	50	0.894	0.497
	100	0.713	0.461
Caffeic acid	5	1.649	0.892
	10	1.937	1.780
	25	1.625	1.417
	50	0.957	1.849
	100	1.370	0.994

*RSD: Relative standard deviation

Table 4. Recovery assay's statistical data of the method (n: 3)

Standards	Concentration in sample (µg/mL)	Amount spiked (µg/mL)	Mean amount found in mixture (µg/mL)	Mean recovery (% ± SD*)	RSD**
Chlorogenic acid	0.04	0.02	0.03	99.795 ± 1.516	1.519
		0.04	0.04	97.638 ± 1.563	1.601
		0.08	0.06	99.528 ± 0.297	0.299
Caffeic acid	0.006	0.003	0.0045	97.260 ± 0.507	0.521
		0.006	0.006	102.092 ± 1.325	1.297
		0.012	0.009	101.268 ± 0.406	0.401

*SD: Standard deviation, **RSD: Relative standard deviation

rectangular cells and is covered by a very thick cuticle. The stomata are rarely observed. The abaxial side of the epidermal layer consists of 8-10 rows of collenchymatous cortex cells and is bordered by the endodermis. The pericycle is in a ring form, consists of fibre-like cells with thick walls and wide lumina. Corks occur to adjoining pericyclic fibers. Phloem is composed of thin-walled cells containing starch grains, sometimes containing sclerenchymatous cells. The pith is heterogeneous. The pith cells with thickened walls surround the xylem and are elongated within the xylem as arms. The arm cells contained very dense starch grains. Thin-walled pith cells contain dense and druse crystals. Dead cells occurred toward the middle of the stem (Figure 4).

Leaf anatomy

The transverse section of the leaf is broadly V-shaped. In the midrib, the upper epidermis consists of single-layered epidermal cells with thickened walls. One-five rows of collenchyma are located under the epidermal layer. Between the main vein and the collenchyma tissue, it is filled is filled with parenchymatous cells. The main vein consists of arc-shaped xylem and phloem. The abaxial side of the phloem is lined with 1-2 layers of bundle sheath-like parenchymatous cells containing dense starch grains in a crescent shape. The walls of the midrib lower epidermal cells are very thick, the lumina of the cells is very narrow compared to the upper epidermal cells. The adaxial side of the lower epidermis is powered with 1-9 rows of collenchyma tissue. The abaxial side of the midrib is more protruding than the adaxial side. The cuticle layer is thinner in the lower

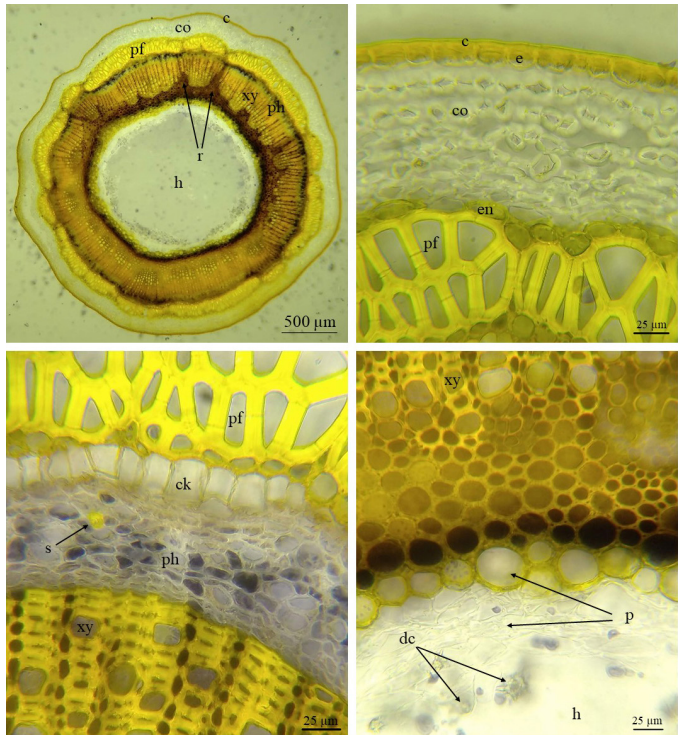

Figure 4. Transverse section of the stem

c: Cuticle, ck: Cork, co: Collenchyma, dc: Druse crystal, e: Epidermis, en: Endodermis, h: Hollow, p: Pith cell, pf: Pericyclic fiber, ph: Phloem, r: Ray, s: Sclerenchymatous cell, xy: Xylem

epidermis compared with the upper epidermis. The stomata are not observed in the midrib, but contain solitary crystals. The leaf lamina is bifacial in the transverse section. The upper epidermis cells are square with a thick adaxial wall and covered by a thick cuticle layer. A palisade parenchyma consists of a single layer of elongated cells. The spongy parenchyma contains thin walled, 1-8 rows of isodiametric cells. Palisade and spongy parenchyma, both contain abundant starch grains. The lower epidermis cells are rectangular, outer walls are thick and smaller than the upper epidermal cells. The cuticle layer is thinner and stomata are observed. Solitary druse crystals are sparse in the leaf mesophyll (Figure 5).

In the leaf lamina surface sections; the stomata were observed only on the lower surface with 3-5 subsidiary cells. The lower epidermal cells are sinuous. The upper epidermis layer is free of stomata and consists of polygonal epidermal cells (Figure 6).

DISCUSSION

In this study, leaves and stem of *L. etrusca* var. *etrusca* were evaluated in terms of its anatomical structures and phenolic compounds.

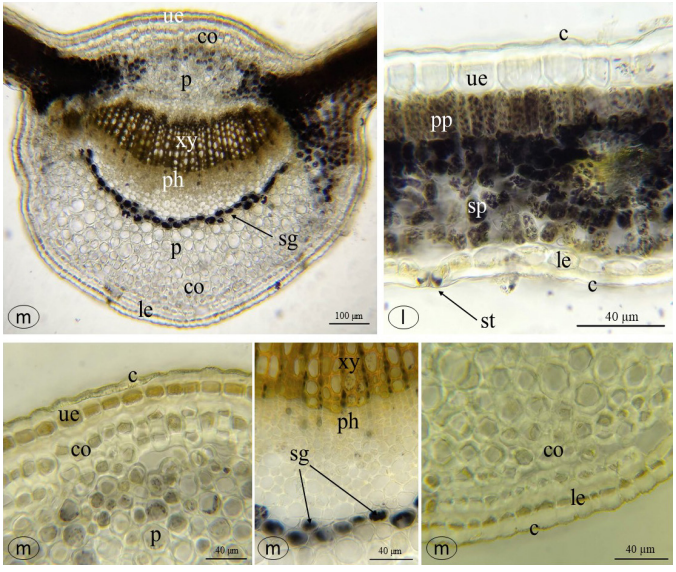

Figure 5. Transverse section of the leaf

c: Cuticle, co: Collenchyma, l: Lamina, le: Lower epidermis, m: Midrib, p: Parenchyma, pp: Palisade parenchyma, ph: Phloem, sg: Starch grains, sp: Spongy parenchyma, st: Stomata, ue: Upper epidermis, xy: Xylem

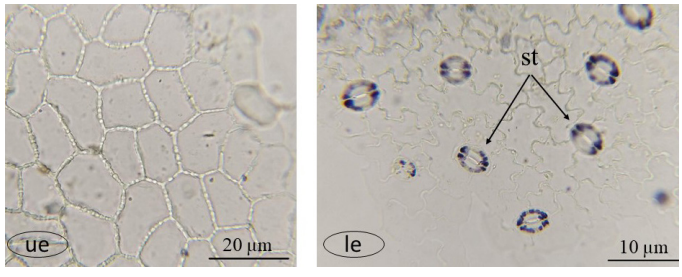

Figure 6. Surface section of the leaf

le: Lower epidermis, st: Stomata, ue: Upper epidermis

HPLC was used for the quantitation of two phenolic compounds, *i.e.* chlorogenic and caffeic acids, and analysis method used was validated. Chlorogenic and caffeic acids have important biological activities such as antioxidant and anti-inflammatory activities.[18,22] Since some *Lonicera* species are used for medicinal purposes, *L. etrusca* var. *etrusca* has also been evaluated in terms of its phenolic compounds with biological activity. The results of HPLC analysis, while amount of chlorogenic acid in the stem and leaf was 1.043% and 1.148%, respectively; the amount of caffeic acid was 0.156% and 0.073%, respectively. It has been determined that chlorogenic and caffeic acids are among the major phenolic compounds in the stem and leaves of *L. etrusca* var. *etrusca*. Phenolic compounds were also detected in some studies with other species (*L. japonica*, *L. confusa*, *L. fulvotomentosa*, *L. macranthoides*, and *L. hypoglauca*). In these studies, it was determined that the amounts of chlorogenic and caffeic acids were similar and chlorogenic acid was higher than caffeic acid.[14,15] These results are consistent with the results of our study.

The results of the anatomical study showed that the transverse section of the stem is hollow, disc-like, and broadly grooved. Epidermal tissue consisting of a single layer of cells is covered with a thick cuticle and rarely stomata are observed. The cortex of stem is characterized by collenchymatous cells and bordered by endodermis. The pericyclic layer is ring-shaped and consists of sclerenchymatous cells with wide lumina and thick-walled. The cork is adjacent to the pericycle layer. Vascular tissue is embedded in the sclerenchymatous pith cells. Phloem sometimes contains sclerenchymatous cells. The pith cells branch out between vascular tissue and starch grains are dense in the pith cells. Thin-walled cells of heterogeneous pith contain dense solitary druse crystals. The anatomical features of stem for *Lonicera* genera were reported by Metcalfe and Chalk[23] as cork usually arises in the pericyclic region with wide luminal and thick-walled cells, the pericycle contains wide fibers or fibre-like elements with thin walls and very wide lumina, phloem includes thick-walled fibers and xylem is in the form of a continuous cylinder, cluster crystals present. These reports are similar to the findings of our study. *L. etrusca* var. *etrusca* leaf is characterized by bifacial lamina, single-layered epidermis, palisade parenchyma with elongated, 1-row cells, spongy parenchyma with 1-8 rows of isodiametric cells, abundant starch grains, and sparse solitary druse crystals. The lower surface includes stomata with 3-5 subsidiary and epidermal cells are sinuous. The upper epidermis layer is free of stomata and consists of polygonal epidermal cells. Leaf features of the Caprifoliaceae family are stated by Metcalfe and Chalk[23], so dorsiventral leaf, a single layer of palisade, and solitary crystals are the same anatomical features determined as a results of our study. This report indicated that the family has Ranunculaceous stomata, whereas our study showed that *L. etrusca* var. *etrusca* has anomocytic stomata.

CONCLUSION

In this study, stem and leaf anatomy of *L. etrusca* var. *etrusca* was studied and the important characteristics were identified.

The transverse section of the stem looked disc-shaped and hollow. The pericycle consists of fibre-like cells with thick walls and wide lumina and the cork occurs adjoining pericyclic fibers. The leaf anatomical structure is bifacial, the stomata located characterized by on the lower surface with 3-5 subsidiary cells. The mesophyll contains solitary druse crystals. Additionally, the amount of chlorogenic acid and caffeic acid, which are the major compounds, were determined by HPLC. Our results follow studies on other *Lonicera* species. Method validation was performed to determine the reliability of the method. The anatomy study coincides with the anatomical features of the genus *Lonicera.* It is important to evaluate these species in terms of anatomical structures and chemical contents, since it is widely grown in our country and is a variety. This study may help minimize confusion between *L. etrusca* var. *etrusca* and other *Lonicera* species, and may support in taxonomically classification for *L. etrusca* var. *etrusca*.

Ethics

Ethics Committee Approval: Not necessary.

Informed Consent: Not necessary.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: D.Ç.P., M.M.H., Design: D.Ç.P., M.M.H., Data Collection or Processing: D.Ç.P., M.M.H., Analysis or Interpretation: D.Ç.P., M.M.H., Literature Search: D.Ç.P., M.M.H., Writing: M.M.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Lentinus squarrosulus Mont. Mushroom: Molecular Identification, *In vitro* Anti-Diabetic, Anti-Obesity, and Cytotoxicity Assessment

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ABSTRACT

Objectives: Mushrooms are fungi with nutritional and health benefits. *Lentinus squarrosulus* Mont., an edible fungus, has traditional usage and relevance in local therapy for managing metabolic diseases. In that view, this study aimed to evaluate the *in vitro* anti-obesity, anti-diabetic, and cytotoxic potential of the chloroform/methanol extract (CME) and aqueous extract (AE) of the mushroom.

Materials and Methods: *L. squarrosulus* was identified using molecular biology tools. The CME and AE were obtained sequentially and, then, subjected to α-amylase, α-glucosidase, and lipase inhibitory enzyme assays as well as total phenolic content (TPC) and flavonoid content (TFC) estimations. The cytotoxic potential of extract fractions of *L. squarrosulus* was assessed using the brine shrimp lethality assay.

Results: The molecular identification of the mushroom displayed that the internal transcribed spacer sequence was an equivalent match to that of *L. squarrosulus* with a high percentage similarity, and thus assigned a unique accession number (KT120043.1). The CME of *L. squarrosulus* had higher TPC, TFC, and α-glucosidase inhibitory activity than AE. Furthermore, AE of the mushroom showed a higher lipase inhibitory potential with an IC_{50} value of 22.28 ± 0.65 µg/mL than the CME, while that of the reference, *i.e.* orlistat was 2.28 ± 0.34 µg/mL. However, these extracts exhibited very low or no α-amylase inhibitory and cytotoxic activity at the tested concentrations.

Conclusion: This study reveals that CME of *L. squarrosulus*, rich in polyphenols and flavonoids, possesses considerable α-glucosidase and lipase inhibitory activities.

Key words: *Lentinus squarrosulus*, lipase, α-Glucosidase, α-Amylase, cytotoxicity

INTRODUCTION

Diabetes mellitus (DM) is a health disease that results from an impairment in the secretion of insulin and some unavoidable degree of resistance to insulin in the periphery, leading to constant hyperglycemia. The chronic form of this hyperglycemia can disrupt protein, lipid, and carbohydrate metabolism leading to serious health concerns. Affecting about 90% of diabetics globally is the type-2 form of the disease (T2DM), making it the most common.[1] A steady rise in cases and diabetes prevalence has been recorded over the past few decades. More than 422 million people are diabetic around the world, and diabetes causes the deaths of about 1.6 million people every year.[2] α-Amylase and α-glucosidase are sugar-hydrolyzing complex enzymes that are mainly secreted from the pancreas and the intestinal chorionic epithelium, respectively. Inhibiting these enzymes is an approach for T2DM therapy as well as reducing postprandial glucose levels, since it can prevent excess glucose absorption by decreasing the rate of carbohydrate breakdown.[3] This metabolic disease also has a close relationship with obesity.[4] Obesity is defined as an abnormal or excessive accumulation of

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massive body storage fats, which may be caused by a mismatch between the rate of intake and expenditure of energy.[5] The number of obesity and overweight cases is increasing and if these trends continue, it is estimated that 2.7 billion adults will be overweight, over 1 billion people will be affected by obesity, and 177 million adults will severely be affected by obesity by 2025.[2] Furthermore, an increase in the storage of lipids in the pancreas can instigate the abnormal functioning of the insulin-producing pancreatic β-cells, which may result in T2DM.[6] The major enzyme in lipid digestion is pancreatic lipase. Its role is to facilitate the absorption of dietary fats by catalyzing the hydrolysis of triacylglycerol into free fatty acids and monoacylglycerol in the lumen of the intestine.[7] Research interest on inhibitors of pancreatic lipase activity has received much attention, possibly due to their anti-obesity activity by delaying the lipid breakdown process.[8]

Mushrooms are widespread. They are fungi visible with definitive basidiocarps (fruiting bodies). Edible mushrooms have served as food supplements and source of bioceuticals because they possess a myriad of compounds that elicit biological activities and play vital roles in human nutrition and health. *Lentinus squarrosulus* mushroom has been reported to be commonly eaten with medicinal properties. *L. squarrosulus* is edible and belongs to the Polyporaceae family. The fruiting body, known as the basidiocarp, is morphologically characterized by a whitish-greyish surface with conspicuous squamules.[9] *L. squarrosulus* usually exists in old or fallen tree trunks, and buried or exposed roots of decaying deciduous trees in some tropical rain forest regions of Africa (Nigeria) and Asia. It usually grows in cespitose clusters of up to three to six basidiocarps.[10] In Nigeria, *L. squarrosulus* is popularly known as "*Ero atakata"* in South-Eastern part[11], and "*Olu-awo, erirokiro, or osun two*" in South-Western part.[12] In traditional medicine, it is used in ulcer treatments,[13] alleviate anemic symptoms, decrease the chances of infertility in both men and women[14], and lower the risk of metabolic diseases.[15,16] Previous studies have indicated that *L. squarrosulus* contains phenolics, tannins, saponins, flavonoids, alkaloids, terpenes, quinolones, and anthraquinones.[17] It has also been reported to possess antimicrobial,[18] immunomodulatory,[19] antioxidant, anticancer, and antihyperglycemic activities.[13,20]

Currently, synthetic drugs are available for α-amylase and α-glucosidase inhibition and treatment for obesity, but the associated undesirable side effects such as bloating, abdominal discomfort, flatulence, and emesis, insomnia, myocardial infarction, and constipation have rendered them less attractive as therapeutic agents.[21] A natural remedy that will be effective, inexpensive, and relatively safe is desirable.

To overcome these short-comings combined with the purported therapeutic effects of the mushroom in traditional medicine, the investigation became imperative. Hence, this study sought to assess antidiabetic, anti-obesity, and cytotoxicity potentials of *L. squarrosulus* as well as identify the mushroom using molecular techniques.

MATERIALS AND METHODS

Reagents and chemicals

Sodium hydroxide (NaOH) solution (2 M), 1% w/v starch, 3, 5-dinitrosalicylic acid (DNSA), potassium sodium tartrate tetrahydrate ($C_4H_{12}KNaO_{10}$), 3% methanol, acarbose, phosphate buffer (20 mM, pH 6.9), DNSA solution (96 mM), maltose standard solution 0.2% (w/v), α-amylase (*Aspergillus niger*) sodium bicarbonate (Na_2CO_3), *p*-nitro phenyl glucopyranoside (pNPG), *p*-nitrophenol, α-glucosidase enzyme (*Saccharomyces cerevisiae*), orlistat, porcine pancreatic lipase enzyme, *p*-nitrophenyl butyrate (pNPB), dimethyl formamide, sodium chloride (NaCl), ethanol, isolation buffer (Tris-EDTA), RNAse, polyvinylpyrrolidone, ITS1 and ITS4 primers, Taq DNA polymerase, *Taq* buffer, DNA template, sterile water, dNTPs-mix and ethidium-stained agarose gel. All other chemicals and reagents used were of quality analytical grade and procured from commercially available sources.

Mushroom sample collection and morphological identification

The mushroom sample collection was carried out on the basis of the reported morphological features and characteristics of the mushroom (*L. squarrosulus*) as described in mycological treaties.[9,10,22,23] The fruiting bodies of the mushroom were harvested from the University of Ibadan (Figure 1).

Molecular identification of a mushroom sample (L. squarrosulus)

Extraction of the genomic DNA

The total DNA of the genome was extracted from the fruiting body of the mushroom using a plant/fungi DNA isolation kit (Norgen Biotek Corporation, Thorold, ON, Canada) strictly following the manufacturer's instructions for use. The DNA after extraction was then stored at -20°C until required.

Polymerase chain reaction (PCR) amplification of the genomic DNA

Amplification of the extracted genomic DNA of the mushroom sample was performed by PCR using ITS1 (5'-*TCCGTAGGTGAACCTGCGG*-3') and ITS4 (5'-*TCCTCCGCTTA TTGATATGC*-3') primers. The reaction involved the use of Taq polymerase "Ready to Go" mixture (Pharmacia, Sweden), the primers and DNA template solution. A GenAmp PCR System 2400, Perkin-Elmer, USA was used to achieve 30 cycles of denaturation at 95°C for 30 seconds; primer hybridization/ annealing at 50°C for 1 min; and primer extension at 72°C for

Figure 1. Harvested fruiting bodies of *Lentinus squarrosulus* Mont.

another 1 min. The products after amplification were subjected to gel purification and electrophoresis on ethidium-stained agarose gel (0.7%). The products after that were directly sequenced and aligned using CLUSTAL W.[24] The data obtained from the aligned sequences were used to plot a dendrogram tree using Molecular Evolutionary Genetic Analysis 4 software.

Extraction of L. squarrosulus samples

The collected mushrooms were freeze-dried and ground to coarse powder with the use of an electric blender. The powdered mushroom (200 g) was weighed and macerated in chloroform/methanol (1:1) at 70°C for 24 h in a sonicator. It was then filtered, and the filtrate was collected and concentrated to obtain the chloroform/methanol extract. The residue was dried and macerated in distilled water at 70°C for 24 h in a sonicator. The extract from the residue was filtered and lyophilized to obtain the aqueous extract.[25,26] The weights of the chloroform/methanol and aqueous extracts were recorded and the different percentage yields (%) calculated.

In vitro antidiabetic activity

Evaluation of α-amylase inhibitory activity of L. squarrosulus extracts

A reported method[25] was used to determine the inhibition of α-amylase activity of the chloroform/methanol and aqueous extracts of *L. squarrosulus*. Five graded concentrations of the extracts and acarbose (104.19-1667 µg/mL) were obtained through two-fold serial dilution in phosphate buffer (20 mM, pH 6.9). Five hundred microliters (500 µL) of the extracts/acarbose were dispensed into well-labeled test tubes, and 500 µL of 2 units of α-amylase solution was added. The resulting mixture was pre-incubated for 15 min at a temperature of 35°C followed by the addition of 500 µL starch solution (1%) to initiate the reaction. The reaction mixture was further incubated for 5 min at a temperature of 25°C. Finally, 500 µL of the colour reagent (96 mM DNSA and 5.31 M sodium potassium tartrate in 2 M NaOH) was added to terminate the reaction and tubes were incubated inside the water bath at 80°C for 15 min. The test tubes were removed and made to cool on ice; thereafter 4.5 mL of distilled water was added to dilute the reaction mixture. Two hundred microliters (200 µL) each of the content in the test tubes were measured into a 96 well microtiter plate and the absorbance read at 540 nm. The concentration of maltose formed was extrapolated from a maltose standard curve. Positive control (containing serially diluted acarbose) and negative control tubes (phosphate buffer) were also included.

Evaluation of α-glucosidase inhibitiory activity of L. squarrosulus extracts

A previously reported method was used.[25] The mushroom extracts and acarbose were prepared in phosphate buffer (pH 6.9). Five graded concentrations of the mushroom extracts and acarbose (78.13-1250 µg/mL) were determined by a two-fold serial dilution in test tubes. One hundred (100 µL) of α-glucosidase enzyme (1 U/mL) was pre-incubated with 50 µL of the varying concentrations of the mushroom extracts for 10 min at room temperature. Then, the reaction was started by addition of 50 µL of 3 mM pNPG. The reaction mixture was brought to a stop by the addition of 2.5 mL of 0.1-M Na_2CO_3 solution after incubation at room temperature for 20 min. 200 µL each of the content in the test tubes were dispensed into a 96 well microtiter plate and the enzymatic activity of α-glucosidase was after that determined by measuring the amount of the yellow *p*-nitrophenol released from the pNPG at 405 nm on the SPECTRAmax Gemini XS microplate reader. Positive control (containing serially diluted, acarbose) and negative control tubes (phosphate buffer) were also included.

In vitro anti-obesity activity

Evaluation of anti-lipase activity of L. squarrosulus extracts

Pancreatic lipase inhibition activity of the mushroom extracts was determined using pNPB as the substrate. The effect of the mushroom extracts on lipase activity was determined using a modified method described.[27] The mushroom extracts and orlistat were prepared in phosphate buffer (pH 6.9). Two-fold serial dilution in test tubes were used to obtain five graded concentrations of the mushroom extracts and orlistat (78.13-1667 µg/mL). The mushroom extracts and orlistat (50 µL) were then, pre-incubated with 50 µL of pancreatic lipase for 1 h in the reaction buffer at room temperature. pNPB (1 µL) was added to initiate the reaction followed by further incubation of the reaction mixture for 5 min at the room temperature (29°C). The amount of *p*-nitrophenol released in the mixture was estimated at 405 nm using a ultraviolet-visible (UV-Vis) spectrophotometer (SPECTRAmax Gemini XS, Molecular Devices, USA). Positive control (containing serially diluted orlistat) and negative control tubes (phosphate buffer) were also included.

Cytotoxicity assessment

The cytotoxicity assessment of *L. squarrosulus* extracts was carried out using the brine shrimp lethality assay. The eggs of *Artemia salina* (brine shrimp) were obtained from an aquarium shop, UK. The brine shrimp eggs were hatched in natural seawater (from Bar Beach, Lagos) contained in a small reservoir tank, under adequate illumination for 48 h. The hatched nauplii (larvae) were attracted to the illuminated side of the tank and collected with a pasteur pipette. Ten of the brine shrimps (nauplii) were transferred into each extract at selected varying concentrations (5 dilutions, 1.6-5.0 µg/mL) in tubes. Cyclophosphamide was used as the positive control. After 24 h, the number of surviving naupliis were counted. The percentage mortality of each concentration and LC_{50} were calculated and compared with the control. Data were carried out in triplicate. Crude extracts were considered toxic, when their LC_{50} values were less than 100 µg/mL.[28]

Phytochemical composition of L. squarrosulus extracts

Determination of total phenolic content (TPC)

TPC of the chloroform/methanol and aqueous extracts of *L. squarrosulus* was assessed using the Folin-Ciocalteu's reagent using a previously reported method.[29] Briefly, 0.1 mL of mushroom extract was mixed with 0.1 mL of Folin-Ciocalteu's reagent (1:1, v/v) and incubated for 5 min. 1 mL of 7% sodium carbonate (Na_2CO_3) solution was added to the mixture and distilled water was used to make up the volume of the mixture

to 2.5 mL. This was mixed thoroughly and kept in the dark for 90 min at room temperature. Absorbance was measured using a UV-Vis spectrophotometer at 750 nm against the reagent blank. TPC was expressed as gallic acid equivalent (GAE)/g of dry matter based on the standard curve.

Determination of total flavonoid content (TFC)

TFC of the mushroom extracts was determined using the aluminum chloride colorimetric method.[30] The mushroom extract solution, 0.3 mL of 1 mg/mL, was added to a mixture containing 3.4 mL of 30% methanol, 0.15 mL of 0.3 M sodium nitrite ($NaNO_2$), and 0.15 mL of 0.3 M aluminum chloride ($AlCl_3.6H_2O$). The resulting mixture was incubated for 5 min at room temperature. Then, 1 mL of 1 M NaOH was added to the mixture. The absorbance of the reaction mixture was measured against the blank at 506 nm using a UV-Vis spectrometer. TFC, expressed as mg rutin equivalent (RE)/g of the dry matter, was calculated from the calibration curve.

Statistical analysis

IC_{50} /LC_{50} values are expressed as mean ± standard error of mean of the three independent values. The values were compared using the Mann-Whitney *U* test. *p* values of <0.05 were considered a statistically significant difference.

RESULTS

Molecular identification of L. squarrosulus

The internal transcribed spacer (ITS) region sequence of the rDNA was used to identify the mushroom. The BLAST analytic result from Genbank revealed that the ITS sequence of the mushroom sample matched that of *L. squarrosulus*. with a unique accession number (KT120043.1). The dendrogram tree for *L. squarrosulus* is shown in Figure 2.

Extract yield of L. squarrosulus

The percentage yields of CME and AE of *L. squarrosulus* mushroom were 3.72 and 9.10%, respectively (Table 1).

Phytochemical analysis of L. squarrosulus extracts

TPC and TFC

The results of the TPC and TFC of the CME and AE of *L. squarrosulus* are presented in Table 1. From the table, the CME (239.92 ± 0.65 mg GAE/g sample) showed higher TPC than the AE (220.75 ± 0.34 mg GAE/g sample). Similarly, TFC of AE (217.43 ± 0.85 mg RE/g sample) was less than that of CME (348.86 ± 0.32 mg RE/g sample). However, both extracts of *L. squarrosulus* possessed high TPC and TFC.

Inhibition of α-amylase, α-glucosidase and lipase activities of L. squarrosulus

Both extracts (CME and AE) of *L. squarrosulus* exhibited very low α-amylase inhibitory activity at the tested concentrations with IC_{50} values of >1670 µg/mL compared to that of the standard, acarbose with an IC_{50} value of 726.49 ± 1.66 µg/mL (Table 2). The CME of *L. squarrosulus* showed better inhibitory activity with an IC_{50} value of 451.13 ± 2.14 µg/mL than the AE (IC_{50} >1250 µg/mL) against α-glucosidase (Table 2). Although, it is not as active as acarbose with an IC_{50} value of 235.51 ± 1.34 µg/mL against α-glucosidase (Table 2). Furthermore, CME and AE exhibited lipase inhibitory activity with IC_{50} values of 28.11 ± 1.37 and 22.28 ± 0.65 µg/mL respectively. However, the orlistat had the best lipase inhibitor activity (IC_{50}: 2.28 ± 0.34 µg/mL).

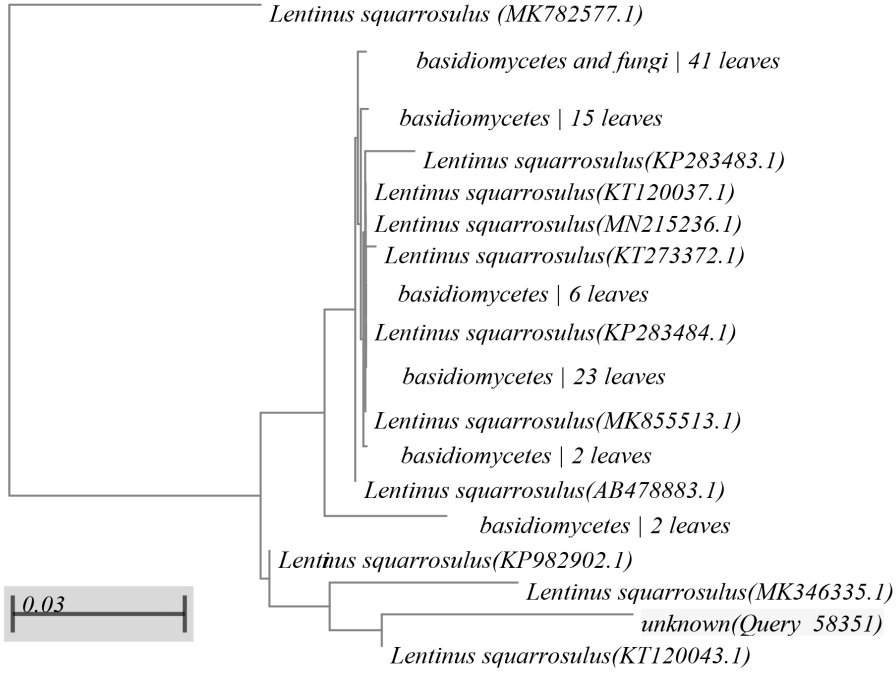

Figure 2. Dendrogram tree of *Lentinus squarrosulus* Mont. from phylogenetic analysis

Table 1. Percentage yield, total phenolic content and total flavonoid content of *Lentinus squarrosulus* extracts

The extracts	Percentage yield (%)	TPC (GAE/g sample)	TFC (mg RE/g sample)
CME	3.72	239.92 ± 0.65	348.86 ± 0.32
AE	9.10	220.75 ± 0.34	217.43 ± 0.85

TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalent, RE: Rutin equivalent, CME: Chloroform/methanol extract, AE: Aqueous extract

Table 2. Cytotoxicity assessment, α-amylase, α-glucosidase, and pancreatic lipase inhibitory activities of *Lentinus squarrosulus* extracts

	IC_{50}/LC_{50} (mean ± SEM) µg/mL		
	The extracts		Standard drugs
In vitro assays	CME	AE	
α-Amylase inhibition	>1670	>1670	*726.49 ± 1.66
α-Glucosidase inhibition	451.13 ± 2.14	>1250	*235.51 ± 1.34
Pancreatic lipase inhibition	28.11 ± 1.37	22.28 ± 0.65	#2.28 ± 0.34
Cytotoxicity assessment	257.10 ± 1.33	251.10 ± 3.59	+15.19 ± 2.00

*Acarbose, #Orlistat, +Cyclophosphamide, SEM: standard error of mean, CME: Chloroform/methanol extract, AE: Aqueous extract

Cytotoxicity assessment

The result of the cytotoxicity assessment using brine shrimp lethality assay revealed that the CME and AE of *L. squarrosulus* had LC_{50} of 257.1 ± 1.33 µg/mL and 251.1 ± 3.59 µg/mL respectively, while cyclophosphamide had LC_{50} value of 15.19 ± 2.00 µg/mL.

DISCUSSION

To harness the numerous potentials that an organism offers, accurate and reliable taxonomy of such an organism is critical to confirm the biological species for various prospective usages. Molecular techniques, which employ the use of DNA barcoding using the ITS region sequencing eliminate the challenge of irregular morphology and possible indiscrimination among macrofungal species, which are often associated and remain a setback with morphological taxonomy. Molecular technique was used to identify *L. squarrosulus* in this study.[31] The ITS rDNA region sequence as a proposition is considered as one of the most essential tools for identifying fungal species that are isolated from environmental and biological sources.[32] This study revealed that the ITS sequences obtained for the mushroom sample compared to those in the database using NCBI-BLAST displayed significant sequences similar to the query sequences used to identify the organism with a high percentage similarity. Hence, the BLAST result identified the mushroom sample as *L. squarrosulus*, with a phylogenetic tree generated to further reveal and confirm the identity of the mushroom species.

In this study, it was observed that the extraction yield of the mushroom in aqueous water is higher than that of the chloroform/methanol. Water or an aqueous solution is more polar than chloroform/methanol, suggesting that the extraction yield increases with an increase in polarity. Similar findings that support the result of this study have been reported.[23] Thus, the extraction yield may depend on one or more of the solvents with different polarities, the time of extraction, temperature, pH, and composition of the sample.

Edible and medicinal mushrooms hold a definite promise as functional food and nutritional supplements to manage DM and obesity because of their rich bioactive components.[33,34] These natural active substances or their primary metabolites consumed in traditional medicine or as nutraceuticals contain antioxidants, fibers, and other phytochemicals that favorably demonstrate both anti-obesity and antidiabetic activities through the modulation and regulation of diverse cellular and physiological pathways. These effects include appetite regulation, modulation of lipid absorption and metabolism, enhancement of insulin sensitivity, thermogenesis, and changes in the gut microbiota.[34]

Disease conditions such as obesity, hyperlipidemia, and T2DM are likely to occur, when there is an imbalance between energy intake and energy expenditure.[35,36] To treat these complications and obesity, the enzymatic inhibition of pancreatic lipase is a very essential path. Dietary fats consisting of triglycerides are hydrolyzed to release fatty acids and glycerol, which are absorbed by the mucosa of the small intestine. In response to food intake, lipase is therefore secreted by pancreas into the small intestine to catalyze the hydrolysis step. The drug orlistat, which is used to treat obesity, inhibits the activity of human pancreatic lipase by forming a bond that is covalently linked with the enzyme at its active site.[34,36] Many of the phytochemically active components, such as flavonoids, saponins, polyphenols, and caffeine have been shown to inhibit pancreatic lipase activity *in vitro* and are comparable to orlistat.[36] In this study, both extracts of *L. squarrosulus* inhibited pancreatic lipase enzyme activity. Mushrooms have been well documented to have anti-obesity effects through various possible mechanisms. A study evidenced that *Lentinus edodes* and *Cordyceps militaris* reduced triglyceride, total cholesterol, plasma glucose, and hypertension

in diabetic rats.[37] *Lentinus strigosus* another species of *Lentinus*, was reported to possess anti-obesity activity by affecting the food intake and locomotion of *Caenorhabditis elegans*.[38] In this study, *L. squarrosulus* exhibited anti-obesity effects through inhibition of pancreatic lipase activity. *Pleurotus sajor-caju* and *Adiantum capillus-veneris* have also shown similar anti-obesity activity through the same mechanism in addition to lowering total cholesterol, triglycerides, and atherogenic index.[5,39] These observed activities are attributed to the presence of bioactive components such as saponins, flavonoids, and polyphenols.

DM is a consequence of unequilibrated insulin production and/or insensitivity to the effect of this hormone in signal transduction of cellular receptors. Most of the T2DM complications are due to hyperglycemia as their main cause.[4] One of the effective strategies for T2DM management is the inhibition of complex polysaccharide hydrolysis by pancreatic α-amylase and absorption limitation of glucose by inhibiting intestinal α-glucosidase. Mushrooms have antidiabetic properties with different mechanisms of action. β-Glucans and their enzymatically hydrolyzed oligosaccharides from the mushroom *Agaricus brasiliensis* show antiarteriosclerotic and antihyperglycemic activities, indicating antidiabetic activity in diabetic rats, which corroborates with enhanced insulin secretion from pancreatic islets and proliferation of islets in diabetic or normal rats.[36] The results of our study revealed that the chloroform/methanol extract of *L. squarrosulus* had the highest α-glucosidase inhibitory activity, while the extracts of *L. squarrosulus* did not show any inhibitory effect on α-amylase at the concentrations tested. Stojkovic et al.[37] in 2017 reported antidiabetic activity of methanol extract of *Morchella conica* as another mushroom species that did not inhibit α-amylase activity; but had inhibitory potential on α-glucosidase activity. Moreover, a previous study revealed terpenoids as inhibitors of α-glucosidase.[39] *L. squarrosulus* contains bioactive components such as terpenoids, saponins, and polyphenols amongst others.[40] Hence, the antidiabetic activity of *L. squarrosulus* through inhibition of α-glucosidase activity leading to delayed process of glucose uptake may be attributable to the presence of terpenoids and other bioactive components.

The need to know the toxic potential of edible and medicinal mushrooms is paramount for their safe consumption and usage. The brine shrimp test is a rapid and simple bioassay for testing lethality of extracts as a means of ascertaining their cytotoxic properties. The test system has been proven to be convenient for monitoring the biological activities of products of natural origin.[41] *L. squarrosulus* mushroom extracts appeared less toxic than cyclophosphamide, a standard toxic drug. Hence, it is relatively safe at the tested concentrations.

CONCLUSION

In conclusion, obesity and T2DM are however complex disease conditions, to prevent, treat, and manage these diseases and their complications, a holistic approach is required that involves a combination of factors such as regular exercise, diet modifications, and pharmacotherapy that requires further advancements. Mushrooms, due to their numerous bioactive components and reported therapeutic advantages, appear promising in the search for treating obesity and T2DM. Therefore, the outcome of this study indicates that the chloroform/methanol extract of *L. squarrosulus* mushroom, rich in polyphenols and flavonoids possess considerable α-glucosidase and pancreatic lipase inhibitory activities and appears less toxic. Thus, it might be explored or combined with existing treatments to reduce the prevalence of diabetes and obesity, and their complications.

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Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

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Authorship Contributions

Concept: O.O.A., Design: O.O.A., A.M.A., O.A., Data Collection or Processing: A.M.A., A.N., O.A., Analysis or Interpretation: O.O.A., P.U.E., A.N., T.A.O., Literature Search: A.M.A., O.A., P.U.E., Writing: O.O.A., A.M.A., P.U.E., T.A.O.

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ORIGINAL ARTICLE

Peptide Sequence of Pili Subunit Protein 49.8 kDa *Shigella flexneri* as Antigenic Epitope for Shigellosis Vaccine Development

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ABSTRACT

Objectives: This study investigates the amino acid sequence and identifies antigenic epitopes of 49.8 kilodalton (kDa) pili protein *Shigella flexneri*, which will be used as candidates for the shigellosis vaccine.

Materials and Methods: Our study is a prospectively descriptive laboratory. We used bacterial isolate of *S. flexneri* pili isolation was performed using a pili cutter and sodium dodecyl-sulfate polyacrylamide gel electrophoresis. The amino acid sequences were analyzed using liquid chromatography dual mass spectrometry (LC-MS/MS) method in the proteomic laboratory. The target epitope antigenicity analysis was tested using Kolaskar and Tongaonkar Antigenicity software. The Bepired Linear Epitope Prediction software is used for epitope mapping. PymOL software was used for the visualization of proteins and molecular docking. Peptides and antibodies were applied to hemagglutination test and immune response was tested using the dot blot method.

Results: LC-MS/MS analysis results from the mascot server showed that the 49.8 kDa pili protein is *S. flexneri* similar to the flagellin protein of *S. flexneri* 1235-66 (ID I6H2T2). The results of antigenicity analysis and epitope mapping showed that areas of protein that has the most potential and antigenic epitopes are the regions 98-111 and 263-290 with the amino acid sequences, *QSSTGTNSQSDLDS* (Q-S) and *DTTITKAETKTVTKNQVVDTPVTTDAAK* (D-K). The results of the molecular docking interaction test between the peptide and the B-cell receptor have a low binding energy. Peptide Q-S and peptide D-K antigens are hemagglutinin molecules because they can agglutinate erythrocytes. The immune response between peptide antigens and anti-peptide antibodies can react based on color gradations in the dotblot method.

Conclusion: The amino acid sequences Q-S and D-K are potentially antigenic epitopes. These peptides can be used to develop candidates for shigellosis vaccine.

Key words: *Shigella flexneri*, pili protein, antigenic, epitope

INTRODUCTION

Shigellosis is an acute intestinal infection. The symptoms can range from mild diarrhea to severe inflammation. The characterization of bacillary dysentery is stomach cramps, fever, bloody stools, and mucus, especially in toddlers.[1,2] Infection occurs globally, and in all people of all ages, endemic diseases occur in children aged 1-4 years, especially those living in low- and middle- income areas.[3]

Research conducted by Sumarno et al.[4] shows that in pili *Shigella dysenteriae* contains a molecular weight hemagglutinin protein of 49.8 kilodalton (kDa) adhesin protein. Besides, other proteins are found 7.9 kDa subunit protein, which is an anti-hemagglutinin. Both of these proteins are adhesin molecules in mice enterocytes. The results also revealed that *S. flexneri, S. sonnei* and *S. boydii* were also found to have pili proteins with molecular weights of 49.8 kDa and 7.9 kDa.[5]

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The type and function of the protein *S. flexneri* 49.8 kDa are still unknown. We had to analyze amino acid sequences of the 49.8 kDa pili protein type from *S. flexneri*. Several studies have carried out secondary analysis and identification of epitopes in adhesin proteins as vaccine candidates. Pore et al.[6] conducted research on amino acid sequences on 34 kDa protein *S. flexneri* before recombinant. The results showed that the 34 kDa protein is an OMPA protein from *S. flexneri*. Sharma et al.[7] performed an analysis of modeling prediction of epitopes on OMP protein *S. flexneri* 2a. Research with 3D structural modeling has also been carried out with a 38 kDa protein model, which is the OmpC *S. flexneri* 3a protein.[8] After determining the expectation of the type of protein and the epitope, it is easier to perform cloning to produce recombinant proteins. This study aimed to determine the amino acid sequence and identify antigenic epitopes from BM 49.8 kDa pili protein *S. flexneri*, which we would like to use as candidates for shigellosis vaccine.

MATERIALS AND METHODS

S. flexneri bacteria

Our study is a descriptive study conducted in the laboratory for the identification and exploration of the 49.8 kDa *S. flexneri* pili protein epitope, which is an adhesive molecule and has potential as a shigellosis vaccine candidate. Bacteria used in this study were *S. flexneri*. Bacteria were cultured in Mac Conkey, brain-heart broth and thiaproline carbonate glutamate (TCG) medium.[9] The results of bacterial collection on TCG media were collected and then shaved using a pili cutter. The isolated pili were then electrophorized to monitor the weight molecular of 49.8 kDa protein.

Animal and antigenic peptides

We used 10 male mice (*Mus musculus*) Balb/C 6-8 weeks old, which were obtained from Experimental Animal Laboratory, Faculty of Medicine, Universitas Brawijaya, Indonesia. We used an antigenic peptide chemically produced. We purchased the antigenic peptides through the Apical Scientific Sdn. Bhd (Malaysia) in the form of synthetic peptides.

Amino acid sequence, antigenicity identification and epitope mapping of 49,8 kDa pili protein S. flexneri

We processed an amino acid sequence using the in-gel digestion method and analyzed liquid chromatography dual mass spectrometry (LC-MS/MS). The amino acid analysis was obtained from Mascot Server (Hyperlink). Analysis of the identified antigenic protein was carried out using the approach *in silico* bioinformatics with the Kolaskar and Tongaonkar Antigenicity software (http://www.iedb.org) with value threshold (threshold value) 1.0.[10] Epitope Mapping using Linear Epitope Prediction Bepired with a threshold value (entry) 0.35 of IEDB. The structure of proteins with epitope regions was visualized by software Pyre and PyMOL.[11,12] Visualization of 3D structures resulting from molecular docking between peptides and B-cell receptors (BCR) (PDB ID: 5IFH) used PyMol software (https://pymol.org/2/).

Production of serum antibody pili protein epitope 49.8 kDa S. flexneri

We used 5 mice *per* epitope for antibody production. The dose for immunization of each epitope was 50 µg in a volume of 100 µL. The epitope of pili protein 49.8 kDa *S. flexneri* was emulsified with complete Freud's adjuvant (CFA), and then 100 µL was injected intraperitoneally. Weekly boosts were performed using antigens emulsified with incomplete Freud's adjuvant (IFA) at the same dose. The blood was removed from the heart one week after the last booster. The blood was placed in a sterile tube and centrifuged at 10.000 rpm for five minutes. Then the serum was collected for further examination.

Hemagglutination test

We used two epitopes in the form of synthetic peptides to test for hemagglutination, namely, *QSSTGTNSQSDLDS* (Q-S) and *DTTITKAETKTVTKNQVVDTPVTTDAAK* (D-K) as well as serum antibodies from synthetic peptides produced in mice.

Samples IgG diluted half a series in a well-contained microplate V with a volume of 50 µL each in their wells a dilution solution used in PBS pH 7.4. Furthermore, in each well, a synthetic peptide antigen of 50 µL. We then incubated in a water bath by shaking 60 times a minute at 37°C for half an hour. After our incubation period completed in each well, we added 50 mL of mice's blood cells to a concentration of 0.5%. We read the results of the agglutination inhibition reaction after incubation at room temperature for 1 h. As a negative control, hemagglutination inhibition reaction used pre-serum was employed.[5,13]

Dot blot test

We used two epitopes in the form of synthetic peptides to test for dot blot, namely, Q-S and D-K, as used in the hemagglutination test. The dot blot test with immersed the nitrocellulose membranes was in sterile H_2O for 30 min the membrane, dripped with 50 µL antigen (synthetic peptide), and incubated overnight at 4°C. The membrane with primary antibodies was 50 µL, set for 2 h at room temperature. Secondary antibody was added with 1:500 dilutions in FFB solution, incubated at room temperature for 1 h. Chromogen substrate was added and incubated at room temperature for 30 min. We stopped the reaction by adding H_2O-positive. Quality results are seen on the basis of color gradations.[13] This research has obtained a statement letter from the Ethics Commission of Universitas Brawijaya with letter number of 1192-KEP-UB.

Statistical analysis

No statistical analysis was used in this study.

RESULTS

Characterization of pili 48.9 kDa protein of S. flexneri

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis identified *S. flexneri* pili protein. Pili protein profiles were generated from the first to third cuts of pili proteins, as shown in (Figure 1).

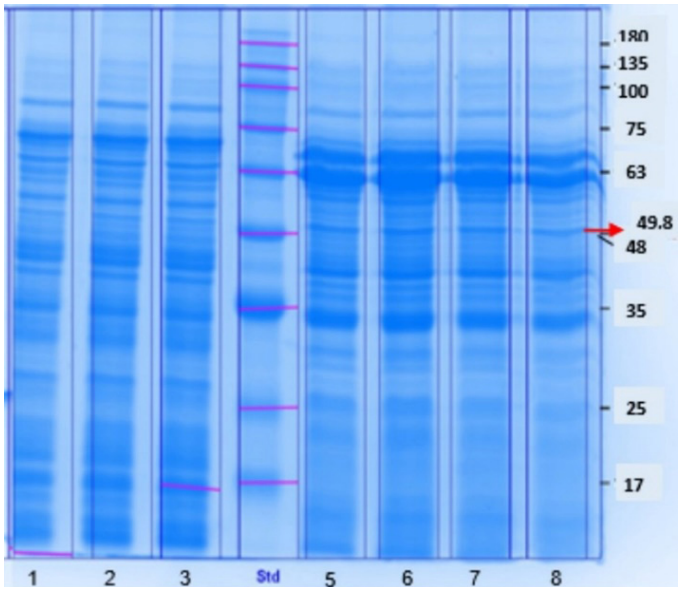


Figure 1. Electrophoresis results in a molecular weight of 49.8 kDa pili protein *Shigella flexneri*. Pili protein profiles have various molecular weights. Columns 1, 2, and 3 results from the 3rd pillar cut; 5 and 6 results from the 2nd pili cut; 7 and 8 results from pili first cut

The analysis amino acid sequence of the protein of 49.8 kDa pili S. flexneri

Analysis Mascot server showed that 49.8 kDa protein *S. flexneri* has homology with flagellin protein belonging to *S. flexneri* 1235-66 (ID I6H2T2), with a query coverage of 18% and a molecular weight of 51.75 kDa (Table 1).

Antigenicity analysis and epitope mapping

Analysis results from antigenicity of protein were done with Kolaskar and Tongaonkar antigenic software (Figure 2A and Table 2). Analysis of epitope mapping used the Bepired Linear Epitope Prediction software shows that some regions have epitopes shown in the yellow area in Figure 2B. Some of these epitopes have high scores as potential antigenic epitopes and areas adhesin molecules in the regions 98-111 and 263-290 with amino acid sequences Q-S and D-K (Table 3; highlight yellow).

Modeling and visualization flagellin proteins

Results of modeling protein structures with antigenic regions and areas of epitopes visualized by Pyrx and PyMO. Based on the visualization results, areas that have potential epitopes are in the order of 98-111 and 263-290. Known areas with antigenic potential at positions 276-283 are potential epitopes in regions 263-296 (Figure 2C, wire; red and yellow).

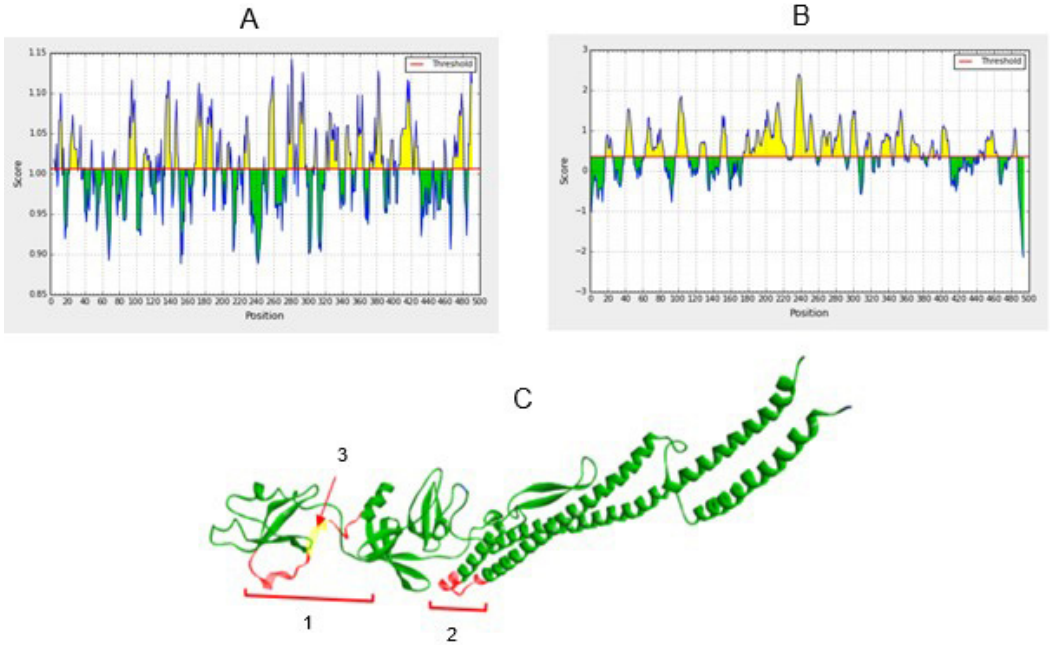


Figure 2. Analysis graph prediction of antigenicity, epitope mapping, and visualization of the *Shigella flexneri* flagellin protein. A) Antigenicity analysis (the yellow graph shows the lively antigenic areas, green shows antigenic negativity); B) Epitope mapping analysis (the yellow graph shows areas with potential epitopes, green indicates negative potential epitopes); C) Visualization of protein structures are characterized by (ribbon; green) with antigenic regions (line; yellow) and epitopes (wire; red). The potential epitope shows in areas C1 (98-111), C2 (263-290) (wire; red), and antigenic C3 (276-283) (line; yellow)

Table 1. Results of amino acid analysis of the 49.8 kDa protein pili using the LC-MS/MS method on mascot server that has homology with the flagellin protein of *Shigella flexneri*

Accession number (uniprot/NCBI)	Protein	Query coverage (%)	MW (Da)	Subcellular location
I6H2T2/ EIQ75074.1	*Flagellin* (*Shigella flexneri* 1235-66)	18	51755	Secreted

MW: Molecular weight, Da: Dalton, LC-MS/MS: Liquid chromatography dual mass spectrometry

Table 2. Analysis of antigenicity flagellin protein used Kolaskar and Tongaonkar software

Protein identification	Accession number	Start	End	Peptide	Length
Flagellin (*Shigella flexneri* 1235-66)	I6H2T2	23	31	*SSLSSAIER*	9
		92	100	*VRELAVQSS*	9
		109	115	*LDSIQAE*	7
		134	141	*GVKVLAKD*	8
		168	178	*LGLDSLSVQDS*	11
		182	189	*TATVVGAG*	8
		225	231	*GQHYVNI*	7
		255	261	*GAVVIGA*	7
		276	283	*KNQVVDTP*	8
		289	296	*AKALVDAG*	8
		325	336	*ALKVDDKYYAAD*	12
		344	350	*AKTVAYT*	7
		356	364	*SKEAAVQFG*	9
		372	388	*IATVGGKQYLASSVKDH*	17
		405	422	*ESPLAKIDAALAKVADLR*	18
		424	429	*DLGAVQ*	6
		469	482	*NILQQAGTSVLAQA*	14

Table 3. Identification of epitopes *Shigella flexneri*'s flagellin protein used bepired linear epitope prediction software

Protein Identification	Accession number	Start	End	Peptide	Length
Flagellin (*Shigella flexneri* 1235-66)	I6H2T2	232	257	*TDSTSTDPGKGNGMYKATIDPDTGAV*	26
		98	111	*QSSTGTNSQSDLDS*	14
		175	224	*VQDSYKTTATVVGAGTYKDGVTITAPT QGEIDAAVGGTAGEGKATVEFKD*	50
		39	49	*NSAKDDAAGQA*	11
		347	358	*VAYTDDKGVSKE*	12
		296	305	*GVTGATDTNT*	10
		263	290	*DTTITKAETKTVTKNQVVDTPVTTDAAK*	28
		149	155	*GANDGET*	7
		232	257	*TDSTSTDPGKGNGMYKATIDPDTGAV*	26
		63	84	*QASRNANDGISIAQTTEGSLSE*	22
		400	409	*ATAKTESPLA*	10
		481	487	*QANQTTQ*	7
		451	465	*SRIEDADYATEVSNM*	15
		313	321	*EDKNGKVID*	9
		331	343	*KYYAADYKDGKIT*	13

Docking molecular visualization and interaction

Two peptides, which were considered as potential epitopes, were predicted for binding interactions between the peptide antigen and B-cell receptor. Molecular docking simulation was performed by interacting BCR-peptide (Figure 3).

Antigenic peptides

From the results of *in silico* analysis of 49.8 kDa protein similar to flagellin protein, we selected two epitopes that were considered potential epitopes. The characteristics of these epitopes are presented in Table 4.

Hemagglutination assay of epitope pili subunit proteins

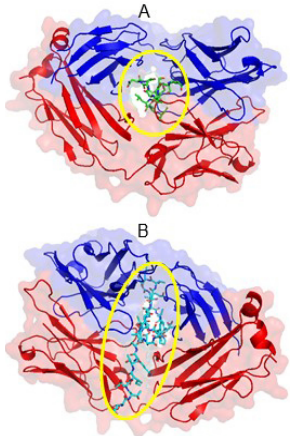
For the antigens' determining ability to agglutinate erythrocytes, we used the hemagglutination test (Figure 4A). The results display the function of anti-hemagglutination test. Antibodies can determine in inhibiting antigens' ability to agglutinate erythrocytes (Figure 4B).

Immune response test using the dot blot method

The dot-blot method results showed that the most effective immune response to peptide Q-S antigen-antibody occurred at 1/500 and 10 ng dilutions (Figure 5A). The most significant result of the immune response to peptide D-K antigen-antibody occurred at 1/1000 and 1 µg dilutions (Figure 5B).

DISCUSSION

The results of the study using a bioinformatics approach to identify antigens in several serotypes of *Shigella* spp. shows the results of the identification of many peptides in *Shigella*



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Peptide	Global Energy	Van der Waals		Hydrogen Bond
		Attractive	Repulsive	
Pep_1	-33,23	-23,66	2,84	-2,94
Pep_2	-42,43	-38,02	18,58	-3,18

Figure 3. Visualization of 3D structure of the molecular docking analysis used PyMol software (https://pymol.org/2/). A. Pep_1 vs BCR; B. Pep_2 vs BCR; C. Value of binding energy peptide and BCR. The yellow circle indicates the location of the peptide when it binds to BCR

Pep_1: *QSSTGTNSQSDLDS*, Pep_2: *DTTITKAETKTVTKNQVVDTPVTTDAAK*

BCR: B-cell receptor

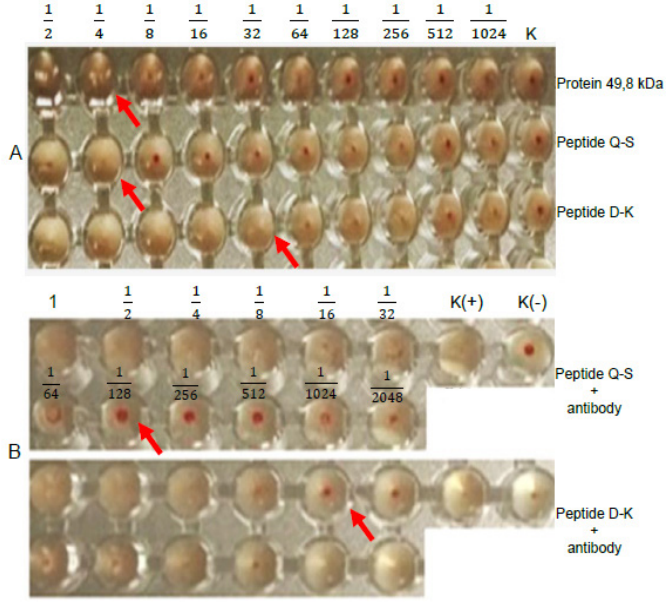

Figure 4. The results of hemagglutination and hemagglutination inhibition examination. A) The dilution used for the hemagglutination test of 49.8 kDa pili protein, Q-S peptides and D-K peptides are 1/2-1/1.024 (positive agglutination is indicated by a red arrow); B) The dilution used for the antihemagglutination test for Q-S peptides and D-K peptides is 1-1/2.048 (positive antiagglutination is indicated by a red arrow)

Q-S: *QSSTGTNSQSDLDS*, D-K: *DTTITKAETKTVTKNQVVDTPVTTDAAK*, K: Control

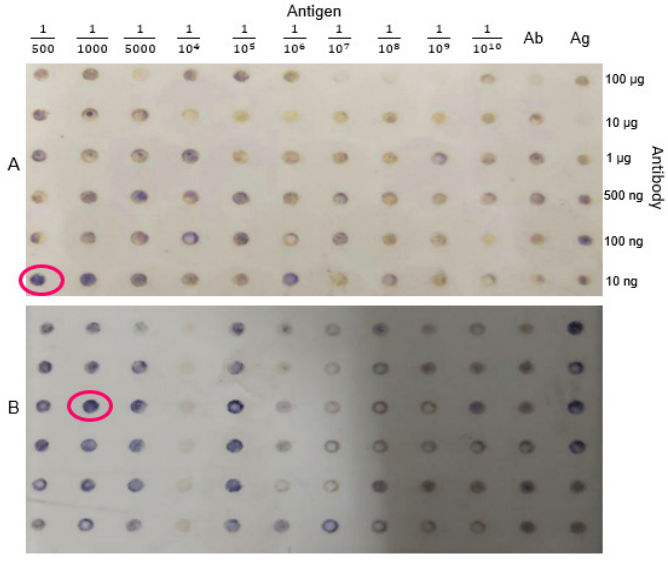

Figure 5. The results of the immune response antigen and antibody used the dot blot method. A) Q-S peptide; B) D-K peptide. More purplish blue indicates the stronger the immune response (red ring)

Q-S: *QSSTGTNSQSDLDS*, D-K: *DTTITKAETKTVTKNQVVDTPVTTDAAK*

Table 4. The characteristics of antigenic peptides determined by *in silico* analysis

Name	Sequence of Peptide (Epitope)	Length	Formula	Purity	Solubility
Peptide 1	*QSSTGTNSQSDLDS* (Q-S)	14	$C_{53}H_{87}N_{17}O_{29}$	Crude	Soluble in water
Peptide 2	*DTTITKAETKTVTKNQVVDTPVTTDAAK* (D-K)	28	$C_{126}H_{218}N_{34}O_{48}$	Crude	Soluble in water

bacteria that are immunogenic.[14] Bioinformatics serves to design vaccine candidates and can also be used to analyze the mechanism of bacterial resistance to drugs.[15]

The profile band of *S. flexneri* clearly displays that it has a molecular weight of 49.8 kDa as an adhesion protein. This indicated that hemagglutinin and an adhesion protein in *S. dysenteriae* and *S. flexneri* have a molecular weight of 49.8 kDa.[4,5] Adhesins are proteins that can attach to cell receptors. This protein can also clump the enterocyte cells.[9]

Pili protein (49.8 kDa) of *S. flexneri* analyzed with LC-MS/MS an in-gel digestion approach. Analysis Mascot server showed that 49.8 kDa protein *S. flexneri* has homology with flagellin protein belonging to *S. flexneri* 1235-66 (ID I6H2T2), with a query coverage of 18% and a molecular weight of 51,75 kDa. Accession number I6H2T2, which is the ID of the uniprot database. We used database uniport because our study is about proteomics. However, the data is the same as the protein in NCBI database with accession number EIQ75074.1 (Table 1). Flagellin is a structural component that helps bacterial motility. This ability helps bacteria to avoid the immune system as well as harmful components in the host.[16,17] As a virulent factor for Gram-negative pathogenic bacteria, flagellin is responsible for several functions such as movement, adhesion, and invasion.[18] *Shigella flagella* (flash) has similarities to flagellin from *Escherichia coli*, *Salmonella* spp., and *Proteus mirabilis*. The results of the study indicate that *Shigella* is capable of forming flagella.[19] The studies on *Shigella* genus revealed that 4 of 12 strains of *S. boydii* have fliC gene as protein-coding flagellin similar to *S. flexneri*.[20]

The analysis of protein antigenicity *in silico* with Kolaskar and Tongaonkar antigenicity software on flagellin proteins showed that the protein is very immunogenic because it has peptide regions that are potentially antigenic. These results follow Utami et al.[9] research, which states that the 49.8 kDa *S. flexneri* pili protein is an adhesin protein that can increase the intestinal immune response of mucosa. Results of analysis epitope mapping *in silico* using Bepired Linear Epitope Prediction software indicated that the protein flagellin epitopes are some regions. The immune system can recognize the epitopes following the yellow area in Figure 2B. After scoring, some epitopes were identified based on antigenic epitopes two, and the most potential is in the regions of 98-111 and 263-290 with the sequences of Q-S and D-K. A study has successfully identified the IpaC protein parts and the IpaD protein; *S. flexneri* 2a, which are epitopes of that protein.[21,22] 3D structural modeling can predict the presence of antigenic peptides or epitopes from the OMP proteins; *S. flexneri* 2a and *S. flexneri* 3a.[7,8]

Based on analysis visualization using Pymol software pointed out that the model of the structure of the flagellin protein with antigenic region peptides in the amino acid sequence D-K appeared to have the same area as the antigenic region and the epitope location. The peptide is considered a potential epitope. Further analysis using software for adhesin prediction showed that the peptide Q-S is an adhesive region. These results support the hypothesis that receptors will recognize the peptide on the surface of the host cell. On the surface of the host cell, there are specific proteins called receptors. The bacterial adhesive can be glycoprotein or lipoprotein found in fimbriae or pili.[23]

We performed docking analysis on peptides with B-cell receptors or BCRs. Our molecular docking studies aimed to determine vaccine candidate peptides that have low binding energies. The docking results displayed that pep_1 and pep_2 had a low average binding energy, which allows the initiation of a biological response, namely, activation of BCR capable of triggering an immune response in B-cells to produce specific antibodies. The PatchDock and FireDock programs are significantly faster and perform slightly better than other programs because they can overcome protein flexibility. Docking applications can be used for polypharmacology prediction, drug use, fishing targets, and profiling.[24-26]

We ordered the antigenic peptides for the *in silico* analysis in the form of synthetic peptides. The pure peptide was used as this is a preliminary study to prove our peptides as potential ingredients of vaccine candidates. So that, we could use protein with higher purity later, if our current results are promising. The amino acid sequences we used are soluble in water, making them easy to dissolve in solvents such as PBS (Table 4). We injected peptides into experimental animals for the production of serum antibodies. Our immunization was administered intraperitoneally using CFA and IFA to facilitate peptide's dissolution in the blood.

Our hemagglutination analyses included two antigenic peptides and serum antibodies from both. Antigenic peptides to test for hemagglutination were, namely, Q-S and D-K. The results of the hemagglutination test showed a difference in erythrocyte agglutination that could be observed from Q-S and D-K peptides. Q-S peptide antigen is capable of agglutination at 1/4 titer. Meanwhile, D-K peptide exhibited agglutination at 1/32 titer. These results proved that Q-S and D-K peptide antigen can bind to erythrocytes or known as hemagglutinin molecules. The peptide antigen used is the epitope of *S. flexneri* bacteria's pili protein with a molecular weight of 49.8 kDa, which is an adhesive protein.[9] The anti-hemagglutinin test carried out used serum Q-S peptide antibodies against Q-S peptides, and D-K peptide antibodies against D-K peptides. Q-S peptide antibody can inhibit Q-S peptide antigen starting at 1/128 dilution. While D-K peptide antibody could inhibit D-K peptide antigen starting from 1/16 dilution. The sediment that occurs at the bottom of the well demonstrated presence of antihemagglutination.[13]

Process immunoblotting analysis used dot blotting.[27] The dot blot test also uses the same two antigenic peptides and antibodies as in the hemagglutination test. Antigen-antibody reaction of Q-S peptide and D-K peptide antibodies by reacting to Q-S peptides and D-K used, the dot blot method. The purplish-blue marked positive dot blot test results between Q-S peptide antibody with Q-S peptide and D-K peptide antibody with D-K peptide. The results of the research using the dot blot method in this study follow the results of previous studies, namely that the synthesis of peptide A-K antigen from the 49.8 kDa *S. flexneri* pili protein can conduct an immune response with its serum antibodies.[13]

The reaction of the peptide with the antibody will cause a color gradation of the dot blot results and quantitatively use Corel photo paint.[28] Our study did not adhesion test between antigen with enterocyte cells as a confirmation method to prove that an antigen is an adhesion molecule, as did Milliana et al.[29], who proved that the 28 kDa OMP of *S. flexneri* is an adhesion protein.

CONCLUSION

Pili protein 49.8 kDa has potential antigenic epitopes, namely, Q-S and D-K peptide. Both peptides are hemagglutinin molecules.

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Ethics

Ethics Committee Approval: This research has obtained a statement letter from the Ethics Commission of Universitas Brawijaya with letter number: 1192-KEP-UB.

Informed Consent: Not necessary.

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Authorship Contributions

Surgical and Medical Practices: K.A., A.T.E., Concept: S.R.P., Design: S.P., Data Collection or Processing: K.A., Analysis or Interpretation: K.A., A.T.E., Literature Search: K.A., Writing: K.A.

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ORIGINAL ARTICLE

The Effects of the COVID-19 Pandemic on the Psychology of the Pharmacy School Stakeholders in Türkiye

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ABSTRACT

Objectives: The state of anxiety seen during the coronavirus disease-2019 (COVID-19) pandemic needs to be evaluated and controlled. Studies indicate that high anxiety reduces students' academic performance. The productivity and scientific studies of academicians have also been negatively affected during the pandemic. Psychological conditions of students and academicians need to be improved as high anxiety levels can affect academic achievement. In this study, the effects on psychological well-being (PWB) of COVID-19 anxiety levels of students and academicians in pharmacy schools in Türkiye have been determined.

Materials and Methods: This research was quantitative. As a data collection tool in the research, a questionnaire consisting of three parts was applied to the academic staff and students of pharmacy schools. After the first part of the questionnaire that aimed to determine general information, there were questions about the pandemic anxiety (PA) and the PWB scales. The universe of the study consists of 1.563 academic staff working in pharmacy schools and 17.101 students in these faculties. The level of significance (α) was determined as 0.05 in the analysis made in the study.

Results: Two hundred fortyseven academicians and 1.698 students participated in the research. Data was analyzed by SPSS ver. 25.0 program. 79% of the academic staff participating in the study were women, while 21% were men; for students, it was 77% and 23%, respectively. Female academicians found to have significantly higher (p=0.001) PA level than males. Female students had significantly higher levels of PA (p=0.000) and PWB (p=0.027) compared to male students.

Conclusion: In the study, PA of academicians was generally lower than students, while PWB was higher. When the relationship between PA and PWB was examined, the relationship between PA and PWB of the academicians was very low and positive; for the students, it was found to be very low and negative.

Key words: COVID-19 pandemic, pandemic anxiety, psychological well-being, pharmacy schools

INTRODUCTION

Fear appears as a reactive emotional state to perceptions of real or potential threat associated with autonomic arousal fluctuations, thoughts about immediate danger, and escape actions. Anxiety accompanies fear, when attempts to deal with a threat are unsuccessful; these two emotions are often experienced together.[1]

The coronavirus disease-2019 (COVID-19) pandemic has become an important problem worldwide since December 2019. The pandemic has affected people of all nationalities, continents, races, and socio-economic groups. Measures such as quarantine of human populations around the world in different ways, closing of schools, transition to distance education, social isolation and curfews have suddenly changed daily lives.[2] As a result, the pandemic has caused states of anxiety such as threats, fear, stress, anxiety, sadness, feeling lonely or anger.[3,4] People's mental health is seriously affected because of social distancing measures.[5] Stigmatization, fear of death, the uncertainty of the course of the disease, and immunological complaints are among the causes of anxiety in

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COVID-19 patients.[6,7] A sudden decrease in social contact can have negative psychological consequences, as forced isolation disrupts the social nature of the human being. This can be seen with the development of severe psychological symptoms, such as increased anxiety, depression, and other psychotic affective disorders.[8]

Psychological well-being (PWB) is generally defined as experiencing more positive emotions and less negative emotions.[9] When the literature is examined, different definitions also stand out. Kammann and Flett[10] expressed PWB as "a cognitive process of life satisfaction". Martin and Rubin[11] drew attention to the link between physical health and a high quality of life. Consequentially, PWB is a concept that includes emotional, physical, cognitive, spiritual, and social processes.[12]

In this study, the effects on PWB of the COVID-19 anxiety levels of students and academicians in pharmacy schools in Türkiye have been determined.

MATERIALS AND METHODS

The study is a screening research and quantitative. Scanning model is a study that collects data to determine certain characteristics of the group to be studied.[13] As a data collection tool in the research, a questionnaire consisting of three parts was applied to the academic staff and students of pharmacy schools. After the first part of the questionnaire that aimed to determine general information, there were questions about the pandemic anxiety (PA) scale and the PWB scale.[14,15]

The universe of the study consists of 1.563 academic staff working in pharmacy schools and 17.101 students in these faculties.[16,17] Two hunred fourty-seven academicians and 1.698 students participated in the research. The data of 7 academic staff, who participated in the study, were excluded from the analysis due to incomplete information. The questionnaire was delivered *via* the internet by asking volunteers to participate in the study.

Before starting the survey application, ethical permission was obtained from the Ethics Committee of Ankara University (dated: 12/04/2021, decision number: 06/61) and the Scientific Research Platform of the Republic of Türkiye Ministry of Health. Following the approval of the ethics committee, research data were collected between 12/04-02/05/2021.

Statistical analysis

The research data were evaluated using SPSS 25.0. The normal distribution assumption was confirmed with Kolmogorov-Smirnov test and it was seen that the data displayed a normal distribution. The descriptive data of the research were evaluated with numbers and percentages. Independent samples *t*-test, paired-samples *t*-test, and one-way ANOVA were used for continuous data. A p value of 0.05 or less in all tests was considered significant. The Cronbach-alpha reliability coefficient for the scales used in the research was calculated separately for academicians and students. The Cronbach-alpha reliability coefficient for the PA scale was 0.840 for the academicians and 0.793 for the students; PWB scale was found to be 0.838 for academicians and 0.899 for students.

RESULTS

COVID-19 anxiety and PWB of academicians and students of pharmacy schools in Türkiye were evaluated with scales, and the results obtained are given below. Demographic information of the participants is shown in Table 1.

From Table 1, it is seen that 79% of academicians participating in the study are women, while 21% are men; in terms of academic titles, research assistants with 32.5% the highest participation, lecturers with lowest 2.5%; in terms of age groups, approximately 40% of the participants between the ages of 31-40, considering other age groups, each had approximately 20%. We observed that lowest participation in terms of professional seniority is 16-20 (10%) and 11-15 (13.3%), respectively, and the other groups are around 25%. The married participants were 60.4%; approximately 50% of them were parents, and 24.2% of their closest family members (such as mother, father, and spouse) had COVID-19 disease.

In this study, 77% of the students were women, while 23% were men; in terms of year at the faculty, the highest attendance was in the 1st year (27.7%) and the lowest in the 5th year (9.3%). In the grouping made by age, it is seen that there is a balanced distribution, although there is relatively less participation at the age of 23 and above. 28.8% of the students stated that their relatives (such as mother, father, sibling) had COVID-19 disease, and 7.5% stated that they had a chronic disease.

Normality tests

To verify the study, the data collected must conform to a normal distribution. Compliance of data with a normal distribution is determined by the test of normality.[18] The normality test of the data in this study was carried out with the SPSS ver 25.0 package program. The normality test consists of three stages.[19] In the first stage, the data were examined formally. In the second stage, we checked whether the skewness and kurtosis values of the data were within acceptable ranges. Finally, in the third stage, the data were analyzed with Kolmogorov-Smirnov test.

In the formal examination, which is the first step of the normality test, the minimum and maximum values, average values, frequency, and standard deviation values of the data were calculated, which are displayed in Table 2.

The data collected in the second stage of the normality test were analyzed in terms of skewness and kurtosis values. These values are important in terms of demonstrating how the available data are positioned on the normal distribution curve. This positioning is a guide to check whether the data conform to the normal distribution. The statistical value range for the 5% confidence interval of skewness and kurtosis is expected to be ± 2.58, and for the 1% confidence interval the statistical value range is expected to be ± 1.96.[20] The skewness and kurtosis values of the research data are given in Table 2. When the results of the skewness and kurtosis tests are examined, it is seen that the data obtained from the PA and PWB scales applied to academicians and students are within 5% confidence interval.

Table 1. Demographic information of the participants

Academicians			
n: 240		**Frequency**	**Percent**
Gender	Female	189	78.8
	Male	51	21.3
Academic title	Research assistant	78	32.5
	Instructor	6	2.5
	Assistant professor	68	28.3
	Associate professor	29	12.1
	Professor	59	24.6
Age	30 and below	49	20.4
	31-40	95	39.6
	41-50	53	22.1
	50 and above	43	17.9
Professional seniority (year)	0-5	59	24.6
	6-10	58	24.2
	11-15	32	13.3
	16-20	24	10.0
	20 and above	67	27.9
COVID-19 sight status in nearby (spouse, mother, father, *etc.*)	Yes	58	24.2
	No	182	75.8
Marital status	Married	145	60.4
	Single	95	39.6
Parenting status	Yes	119	49.6
	No	121	50.4
Students			
n: 1698		**Frequency**	**Percent**
Gender	Female	1306	76.9
	Male	392	23.1
Year at school	1	470	27.7
	2	414	24.4
	3	385	22.7
	4	270	15.9
	5	158	9.3
Age	19 and below	360	21.2
	20	337	19.8
	21	372	21.9
	22	325	19.1
	23 and below	304	17.9
COVID-19 sight status in nearby (mother, father, *etc.*)	Yes	489	28.8
	No	1209	71.2
Chronic illness	Yes	128	7.5
	No	1570	92.5

COVID-19: Coronavirus disease-2019

The final stage of the normality test is Kolmogorov-Smirnov test. In this test, the degree of agreement between the distribution of sample data and the theoretical distribution is examined. The significance level of Kolmogorov-Smirnov test's result value is above 0.05 reveals that the data are suitable for distribution. From the test results, it was determined that all data was significant.

Comparison of academicians and students' data

In Table 3, the responses of academicians and students to the PA and PWB scales are generally compared. As it can be understood from Table 3, pandemic anxieties of academicians are generally lower and their PWB is higher than students. On the PA scale, students stated that they agreed at a higher level compared to the academicians to the statements "Sometimes, I have the feeling that the coronavirus will never end.", "I think, I will not get good health care in case of coronavirus transmission." and "I worry about the curfew/prolongation of the ban." On the PWB scale, the academicians stated that they agree more with the statements "I live a purposeful and meaningful life.", "My social environment supports and rewards me." and "I am optimistic about my future."

Table 2. Descriptive statistics of research data

	Standard error average	SD	Variance	Minimum	Maximum	Average	Skewness	Kurtosis
PA students	0.027	1.090	1.189	1.00	7.00	5.1540	-0.905	1.029
PWB students	0.028	1.142	1.305	0.89	6.22	4.2623	-0.823	0.334
PA academicians	0.072	1.119	1.252	1.67	7.00	4.738	-0.265	-0.583
PWB academicians	0.039	0.614	0.377	3.11	6.22	5.014	-0.443	-0.031

PA: Pandemic anxiety, PWB: Psychological well-being, SD: Standard deviation

Table 3. Responses of academicians and students to PA and PWB scales

Pandemic anxiety	Academicians		Students	
	Average	SD	Average	SD
1- I am worried about getting coronavirus.	5.28	1.674	5.28	1.798
2- The possibility of coronavirus transmission to my family or loved ones worries me.	6.12	1.160	6.30	1.270
3- Sometimes I have the feeling that the coronavirus will never end.	4.75	1.710	5.43	1.679
4- I think I will not be able to get good health care in case of coronavirus transmission.	3.75	1.777	4.41	1.858
5- I hesitate to take routine health services due to the coronavirus.	4.93	1.800	5.11	1.853
6- Due to the coronavirus. I cannot continue my social life as before.	5.93	1.325	6.24	1.364
7- The idea of not being able to access equipment such as masks worries me.	3.14	1.886	3.61	2.014
8- The negative news in the media (visual, written, social) about coronavirus worries me.	4.50	1.795	4.96	1.918
9- I am worried about the curfew/prolongation of the curfew.	4.25	1.907	5.04	2.064
Total	42.64	10.069	46.39	9.815
Psychological well-being				
1- I live a purposeful and meaningful life.	5.68	1.083	3.98	1.983
2- My social environment supports and rewards me.	5.62	0.995	4.64	1.727
3- I participate in activities that I am responsible and love in my daily life.	5.38	1.186	4.73	1.730
4- I actively contribute to the happiness and well-being of others.	5.78	0.842	5.35	1.454
5- I am competent and talented in activities that are important to me.	5.75	0.900	5.04	1.541
6- I am a good person and living a good life.	5.87	0.831	5.15	1.528
7- I am optimistic about my future.	5.28	1.275	4.19	1.907
8- People respect me.	5.78	0.861	5.28	1.468
Total	45.13	5.526	38.36	10.282

SD: Standard deviation, PA: Pandemic anxiety, PWB: Psychological well-being

The total scores of academicians and students from the PA and PWB scales are compared with each other and by gender with the independent two-sample *t*-test.

According to the test results displayed in Table 4, academicians have statistically significantly lower PA levels yet higher PWB levels than students in terms of gender and total scale scores.

Findings of academicians

Since it was determined that the data conformed to the normal distribution in the data analysis by academicians and students, independent two-sample *t*-test was used to compare two independent groups, and ANOVA test was used to determine the differences between the means of three or more independent groups.

Independent two-sample t-test results

Table 5 shows the results of two independent samples *t*-test on the gender, marital status, parenting status, and COVID-19 sight status in nearby (spouse, mother, father, *etc*.) of the academicians.

As can be seen from Table 5;

· Female academicians have a significantly higher (p=0.001) PA level than male academicians.

· Married academicians have a significantly higher (p=0.039) PA level than single academicians.

· Academicians with COVID-19 sight status nearby have a significantly higher (p=0.006) PWB level than those who do not.

· There was no significant difference between the parenting status of the instructors of PA and PWB levels.

ANOVA test results

The results of the ANOVA test regarding the age, academic title, and professional seniority of the academicians are presented in Table 6. According to the test results, there was no significant difference between the groups (p>0.05).

Correlation analysis was conducted to measure the effect of the PA of the lecturers on their PWB; a positive but very low correlation (Pearson correlation coefficient: 0.027) was found.

Findings of students

Independent two-sample t-test results

Table 7 shows the results of independent two-sample *t*-test on students' gender, chronic disease, and COVID-19 sight status nearby (mother, father *etc*.).

As can be seen from Table 7;

· Female students had significantly higher PA (p=0.000) and PWB (p=0.027) levels compared to male students.

· There was no significant difference between the levels of PA and PWB of the chronic disease status and COVID-19 sight status in nearby.

ANOVA test results

The results of ANOVA test for age of the students and the year in the school are revealed in Table 8.

According to the test results;

· There was no significant difference between the ages of the students and their PA and PWB levels.

· While there is no significant relationship between students' years at the school and their PA levels, a significant difference existed among their PWB levels. Students in the

Table 4. Independent two-sample *t*-test results of academicians and students by gender and total scale scores

Female		**n**	**Mean**	**SD**	**Significance (2 tailed)**
PA	Students	1306	47.4786	9.18753	0.000
	Academicians	189	43.7302	9.68856	
PWB	Students	1306	38.6639	10.08141	0.000
	Academicians	189	45.2646	5.40723	
Male					
PA	Students	392	42.7474	10.91258	0.010
	Academicians	51	38.5882	10.50748	
PWB	Students	392	37.3520	10.87448	0.000
	Academicians	51	44.6275	5.97314	
Total					
PA	Students	1698	46.3863	9.81462	0.000
	Academicians	240	42.6375	10.06869	
PWB	Students	1698	38.3610	10.28153	0.000
	Academicians	240	45.1292	5.52552	

SD: Standard deviation, PA: Pandemic anxiety, PWB: Psychological well-being

Table 5. Independent two-sample *t*-test results of academicians regarding gender, marital status, parenting status, and COVID-19 sight status in nearby

Gender		n	Mean	SD	Sig. (2 tailed)
PA	Female	189	43.730	9.689	0.001
	Male	51	38.588	10.508	
PWB	Female	189	45.265	5.407	0.466
	Male	51	44.628	5.973	
Marital status					
PA	Married	145	43.724	10.287	0.039
	Single	95	40.979	9.543	
PWB	Married	145	45.221	5.230	0.752
	Single	95	44.990	5.974	
Parenting status					
PA	Yes	119	42.966	11.005	0.617
	No	121	42.314	9.089	
PWB	Yes	119	45.445	5.179	0.380
	No	121	44.818	5.851	
COVID-19 sight status in nearby					
PA	Yes	58	40.848	11.383	0.120
	No	182	43.209	9.576	
PWB	Yes	58	46.845	4.793	0.006
	No	182	44.582	5.642	

SD: Standard deviation, PA: Pandemic anxiety, PWB: Psychological well-being, COVID-19: Coronavirus disease-2019, Sig: Significance

Table 6. ANOVA test results regarding age, title and professional seniority of academicians

	Age	Title	Seniority
	Significance		
PA	0.277	0.118	0.628
PWB	0.992	0.739	0.958

PA: Pandemic anxiety, PWB: Psychological well-being

1st year have a significantly higher level of PWB than those in the 2nd year, and the students in the 5th year compared to students in the 3rd and 4th years.

Finally, correlation analysis was conducted to measure the effect of PA of students on their PWB; a very weak and negative relationship (Pearson correlation coefficient: -0.068) was determined.

DISCUSSION AND CONCLUSION

In this study, the effects of the anxiety created by the COVID-19 pandemic on PWB of academicians and students in pharmacy schools in Türkiye were determined.

The sudden emergence of COVID-19 virus and the occurrence of a pandemic in a very short time have caused the emergence of situations that affect people's anxiety and PWB. It can be seen that there are many studies on the effect of COVID-19 on human psychology.[21-25] The effects of the pandemic on human psychology have emerged because of the changes and restrictions in the lives of people around the world.

In this study, it was found that academicians generally had lower PA and higher PWB than students. In the literature, the results supporting the fact that PA is lower among the academic staff than in the students have been determined. In a study conducted in England, the mental health of society was examined in the COVID-19 pandemic and the level of anxiety was found to be higher at younger ages.[26] In another study, Joos[27] examined the psychological variables experienced by individuals during the COVID-19 pandemic and found that the anxiety levels of individuals aged 20-30 were higher than those of other ages. In a study investigating the effects of fear and anxiety of COVID-19 on psychological distress, sleep disturbance, and life satisfaction in university students in Vietnam, it was found that fear and anxiety of COVID-19 were positively associated with psychological distress. It has been determined that sleep disorder also positively affects COVID-19 fear and anxiety.[28] Similarly, a study conducted in Italy revealed that COVID-19 seems to be a risk factor for sleep disorders and psychological diseases.[29]

In a study conducted in Iran, the problems faced by academicians during the COVID-19 pandemic period were examined into two

Table 7. Independent two-sample *t*-test results of students on gender, chronic disease, and COVID-19 sight status in nearby

Gender		n	Mean	SD	Significance (2 tailed)
PA	Female	1306	47.479	9.188	0.000
	Male	392	42.747	10.913	
PWB	Female	1306	38.664	10.081	0.027
	Male	392	37.352	10.875	
Chronic disease status					
PA	Yes	128	47.125	10.414	0.376
	No	1570	46.326	9.765	
PWB	Yes	128	38.523	9.7901	0.853
	No	1570	38.348	10.323	
COVID-19 sight status in nearby					
PA	Yes	489	46.775	9.272	0.299
	No	1209	46.229	10.025	
PWB	Yes	489	38.344	10.229	0.965
	No	1209	38.368	10.307	

SD: Standard deviation, PA: Pandemic anxiety, PWB: Psychological well-being, COVID-19: Coronavirus disease-2019

Table 8. ANOVA test results regarding age of students and year in school

	Age	Year in school
	Significance	
PA	0.251	0.262
PWB	0.271	0.000

PA: Pandemic anxiety, PWB: Psychological well-being

groups as those related to university and family. In a study conducted with academicians at Hamadan University of Medical Sciences, it was determined that the most prominent effect of COVID-19 was mental fatigue.[30]

When the total scores obtained from the scales were examined in the study, it was found that female students had a higher PA than female academicians and male students had a higher PA than male academicians. However, the opposite findings were obtained at PWB levels. It is thought that this situation is due to the increase in experience and knowledge gained with age.

Studies have shown that women have higher anxiety levels than men during the pandemic period.[31] In a study conducted in the Philippines, it was found that the levels of stress, anxiety, and depression caused by the pandemic were higher in women. In the same study, it was revealed that single and childless individuals had significantly higher levels of stress, anxiety and depression.[32]

The productivity and scientific research of female academicians have been adversely affected by the pandemic.[33-37] It was also determined in our study that female academicians had a significantly higher (p=0.001) PA level than male academicians. It can be said that this result is compatible to some extent with previous studies showing that women are more prone to mental disorders.[38,39]

In our study, no significant difference was found between the parenting status of the academicians and the levels of PA and PWB. Studies conducted in China and Vietnam have indicated that having children can be significantly associated with mental health problems such as stress, anxiety, and symptoms of depression.[40,41] In a study conducted in Italy, 17% of the participants was observed to suffer severely from fatigue related to their parenting roles, especially during the measures taken by mothers due to the COVID-19 outbreak.[42]

In our study, there was no significant relationship between students' years at the school and their PA levels, but a significant difference was found between their PWB levels. Students in the 1st year have a significantly higher level of PWB than those in the 2nd year, and the students in the 5th year compared to with students in the 3rd and 4th years. A study conducted in the United States indicated that students in the higher classes had higher levels of anxiety.[43] In a study conducted to evaluate the level of anxiety among university students in Sudan, 75% of the students were found to have a low level of anxiety. The results of the same study in Nigeria found that more students experienced moderate to severe anxiety during the pandemic.[31] In a similar study conducted in China, it was determined that students with moderate and severe anxiety levels had a higher rate.[44] In a study conducted in India, it was stated that students' anxiety levels increased more than that of other groups participating in the study during restrictions.[45] Another study conducted in Argentina revealed that being a student causes high psychological distress during quarantine.[46]

Many studies have used broad socio-demographic factors, such as education, age, marital and parenting status, experiences, having children, professional achievements, and health problems, to explain differences in PWB. These experiences vary according to their position in the life course and the nature of the challenge or task posed.[47] Ryff and Keyes[48] found in their study that women scored significantly higher than men on positive relationships and personal growth. Numerous studies have revealed that family and especially marriage has a decisive impact on life satisfaction, PWB, and mental and physical health. Young children and adolescents, who are given love, acceptance, and support by their parents, have higher self-esteem, lower anxiety, and depression levels, more happiness and success, and less behavioral problems.[49]

As a result, the COVID-19 pandemic has affected people of all age groups, although it has occurred to varying degrees. The possibility of getting sick and the uncertainty of the process can cause anxiety and thus a decrease in PWB levels. In this study, PA level among students was higher, but PWB was found to be higher among the academicians in pharmacy schools in Türkiye. This situation may be associated with a higher level of consciousness among the academicians. To increase the PWB of academicians and students during the pandemic process;

· Disclosures made through official authorities should include anxiety-relieving messages, rather than just negative data.

· Accessibility to medical resources and health services should be further developed and improved.

· Digital resources used in distance education should be developed and students' access to course materials should be facilitated.

· Scholarships can be provided to students whose financial situation has deteriorated due to the pandemic.

It is thought that any kind of social support that will be provided in this way will not only reduce the psychological pressure, but will also facilitate the function of seeking help.

Ethics

Ethics Committee Approval: Ethics Committee of Ankara University (dated: 12/04/2021, decision number: 06/61) and the Scientific Research Platform of the Republic of Turkey Ministry of Health.

Informed Consent: Informed consent forms have been obtained from all volunteers.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.Ç., G.Ö., Design: M.Ç., G.Ö., Data Collection or Processing: M.Ç., G.Ö., Analysis or Interpretation: M.Ç., G.Ö., Literature Search: M.Ç., G.Ö., Writing: M.Ç., G.Ö.

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ORIGINAL ARTICLE

Websites as a Gate of Pharmacy Schools to the World: Does National Accreditation Cause a Difference?

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ABSTRACT

Objectives: Websites have become the main information source, being a crucial element of our daily life and a global network. In this context, the importance of the websites of pharmacy schools is considered not only for their educational mission, but also for their gates to the world. In this study, we evaluated the websites of pharmacy schools based on criteria issued in the literature.

Materials and Methods: Websites of all pharmacy schools in Türkiye were evaluated by scoring the availability of predetermined items, where *t*-tests and paired *t*-test were used to compare the groups.

Results: As a result, we found that there is no statistical difference between private and public and accredited or non-accredited schools ($p>0.05$). Also, we observed no statistical difference between the scores in 2012 and 2021 ($p>0.05$).

Conclusion: Most pharmacy schools in this study were public, and the accreditation status still seems challenging. Of these 39 schools, only 14 schools are accredited. Total scores of public schools were found to be higher than the private ones and notably, accredited schools have higher scores. Yet, there is no statistically difference between accredited and non-accredited schools or public and private schools. Importance of websites as a communication tool for higher education institutions (HEIs) is indisputable. In conclusion, pharmacy schools should keep up to date with their official websites, considering the virtual world we fell into with the web technologies' developments. Also, further studies should be conducted on the websites of pharmacy schools regarding user demands. Besides we suggest that pharmacy schools' website' quality should be improved and keeping them up to date should be ensured.

Key words: Faculty websites, national accreditation, pharmacy education, pharmacy school

INTRODUCTION

In today's world, websites are one of the crucial elements of daily life. Also, they became the main information sources and a global network in the last two decades.[1] With the evolution of the internet, a new communication way has taken place and web-based two-way communication has become a more and more leading tool. As a consequence, the structure and quality of websites have come to the agenda and development and degree of quality turned into a question and a research area.[2] Here and now, it is hard to imagine any institution both governmental and non-governmental, without a website. Considering that the internet is an important communication channel, small companies and freelancers that do not have the opportunity to have a website that uses social media accounts for the same purpose. In such an environment, higher education institutions' (HEI) brand image would be built on their web-based communication ability.[3]

In Türkiye, HEIs, which are defined as post-secondary education institutions, are classified as faculties, graduate schools, 4 year schools, conservatories, post-secondary vocational schools and applied science centres by the Council of Higher Education (CoHE). Pharmacy schools count as faculties and they provide a diploma equal to a master's degree to their students.[4] In this context, the importance of the websites of pharmacy schools

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concluded not only for the academic degree that they provide, but also for communication with students and other stakeholders such as other schools or researchers from different parts of the world.

Furthermore, there is an organization for accreditation of pharmacy schools in Türkiye, which is established regarding The Higher Education Quality Council of Türkiye (THEQC). THEQC was founded in 2015 under the "Higher Education Quality Assurance Regulation".[5] Also, considering the quality of higher education, Institutional Accreditation Program has been initiated and an accreditation organization named "The National Society of Assessment and Accreditation of Pharmacy Education" (ECZAKDER) has been established for the accreditation of pharmacy schools.[6-8] The accreditation process begins with the application of a pharmacy school as *per* the requirements determined by ECZAKDER (ver. 5.0). After the pre-evaluation report, applicant pharmacy school was informed about its results. If there is no additional request from ECZAKDER, an audit committee is assigned. Eventually, the compliant school acquires the accreditation approval for 6 years.

Apart from these, the coronavirus disease-2019 (COVID-19) pandemic has transformed the learning process dramatically and nearly all HEI in Türkiye responded to this situation with e-learning platforms, which led to a significant increase in web-based communication. The lockdown and campus closures switched the face-to-face in-person learning to the new world. And websites substituted the billboards in HEIs.[9] In this context, the importance of the websites of all institutions has been recognized once again. Particularly, most of the students could not complete the curriculum and assessments in traditional ways. Those nearing the end of higher education phase have faced various difficulties such as lack of internship opportunities, decrease in job opportunities, being inexperienced in the world of remote work.[10] Moreover, even the internship programs have been completed *via* online internship education programs organized by Turkish Pharmacists' Association (https://www.teb.org.tr/news/9319/TEBGK-Taraf%C4%B1ndan-D%C3%BCzenlenen-Online-End%C3%BCstri-Staj-E%C4%9Fitimi-Program%C4%B1).

Educational institutions' websites and their roles have been the subject of some studies with different aspects.[11,12] However, there is no data on the websites of pharmacy schools in Türkiye. In this study, we evaluated the websites of pharmacy schools based on criteria issued in the literature, to put forward their previous and current status, offer suggestions for their improvement.

MATERIALS AND METHODS

Websites of all pharmacy schools in Türkiye have been evaluated. The list of pharmacy schools was elicited from CoHE's website. We found that 39 schools are providing undergraduate pharmacy education at the beginning of March 2021.[13] Data collection was conducted between 01.03.2021-10.03.2021. The evaluation process was conducted regarding the studies of Gibson et al.[14] and Başok Yurdakul and Coşkun.[11] Also, to compare the characteristics of websites of the schools that existed in 2012, we used the data, presented in 2012, which is prepared by two co-authors of this article. The evaluation criteria and scoring method were adopted from Gibson et al.[14] Relevantly, the availability of each item listed in Table 1 was scored with 1 point. But regarding the hierarchical structure of schools and their scope, the criteria were widened considering Başok Yurdakul and Coşkun.[11] In this context, the content of a website is divided into two main categories: functional and accessibility aspects. Primarily, characteristics such as the information provision and communication networks were assessed as functional aspects. However, characteristics such as using images, the existence of a school logo, and having an independent website were considered as accessibility aspects. The evaluation criteria are given in Table 1.

Additionally, to show the differences between 2012 and 2021, the criteria of the former study were considered a starting point. Except for weekly course program, exam program, curriculum, announcements on the online distance learning process, and social media accounts, other criteria were the same as of 2012. Also, the number of schools has increased since then, so a comparison could only be made with the schools that were established before 2012.

Research hypotheses

The assessment was conducted regarding the national undergraduate pharmacy education accreditation status and public or private schools. By this means, the accreditation status is considered a milestone. Since the aim of the accreditation is stated as "to contribute and facilitate the pharmacy schools' competence in both educational and professional manner", the importance of the website of schools is implicitly emphasized.[7] Furthermore, according to the accreditation standards, it is an expectation from schools, to inform their stakeholders in an exact and accessible way.[15] Moreover, the developments in website usage and its functions with the COVID-19 era and its impact on our lives, websites are considered as the main tool to communicate. Correspondingly, we expect the reflection of these developments on the websites of pharmacy schools. Accordingly, the three hypotheses are designed as follows:

H1: The accredited schools have higher scores than non-accredited schools.

H2: Private schools' scores are higher than public schools' scores.

H3: The current scores of the websites of pharmacy schools those existed in 2012, are higher than their previous scores.

Statistical analysis

Statistical analyses were conducted with IBM SPSS ver. 23 (SPSS, Inc. Chicago, IL, USA). Firstly, descriptive statistics were provided, and a test of normality was conducted. Subsequently, to compare the means of two groups, *t*-test was used to analyze the data. The level of significance was set a priori at $p<0.05$. Furthermore, to compare the scores between 2012 and 2021, paired *t*-test was conducted with the same level of significance.

RESULTS

The total number of pharmacy schools was 16 in 2012, but the number has increased up to 40 in 2021. After the data collection process, a brand new pharmacy school is established, along with the existing 39 schools. Hence, this school was omitted and evaluations were conducted for 39 schools. The status of pharmacy schools that objected to this study is presented in Table 2, with there being on public or private, accredited or non-accredited.[16]

As a result of scoring, the highest score of a school was 23, and the lowest score was 10. The mean of the scores was 16,51. Before comparisons, normality tests are conducted to determine whether the data are modeled by a normal distribution. Since the total data were less than 50, the Shapiro-Wilk test is conducted to test the normality. Both of the comparison groups have a normal distribution (p>0.05).[17] Afterwards, the comparisons are conducted with the t-test and, the results are given in Table 3.

As it is seen in Table 3, there are no statistically significant differences between groups. So, H1 and H2 hypotheses are rejected.

Additionally, the normality test is carried out for data that were obtained for the comparison of the schools that existed in 2012. It is found that the data have a normal distribution regarding the Shapiro-Wilk test (p>0.05). Subsequently, the comparison for scores which are objected to the same schools but different years, the paired t-test was conducted. The results are given in Table 4.

There is no statistically significant difference between the scores in 2012 and 2021 (p>0.05) and, H3 is rejected (Table 4).

Table 1. Evaluation criteria and scoring method

Functional aspects	Accessibility aspects
Corporate history	Having independent website
Mission and vision	Usage of images
Information on administrative board	Sitemap
Search engine	Having language option (*e.g.*, English)
Academic staff	Webmaster
Administrative staff	Updating info
Information on departments	Social media accounts (if applicable)
Contact information	
Academic calendar	
Announcements	
Having menu tab	
School logo	
Employment opportunities	
Weekly course program	
Exam program	
Curriculum	
Announcement on the online distance learning process	

DISCUSSION

In the age of communication technologies, the use of websites in different fields, from travel to education is irreplaceable. For this reason, websites are an important resource for educational institutions to reach their stakeholders to whom they provide services and training.[18,19] Furthermore, visibility, which gives clues about the functioning of an organization, is also an important factor for institutions such as The SCImago Institutions Rankings.[20] In this context, the current status of the pharmacy schools' websites in Türkiye is revealed in a framed perspective in this study.

As shown in Table 2, most schools are public and besides, the accreditation status still seems challenging. According to the mean values of schools, accredited schools have higher scores (17, 21) than non-accredited schools (16.12), which is similar also for public (17.93) and private schools (13.33). However, there were no significant statistical differences between the two groups. Therefore, all the three hypotheses are rejected (p>0.05).

Recently, across the world, HEI has become market-oriented progressively. Hence, creating a brand and proving the quality is crucial for HEI, and accreditation is a distinct element in this context.[21] However, current accreditation standards have

Table 2. Status of pharmacy schools

Status	Accreditation		Total
	Accredited	Non-accredited	
Public	11	16	27
Private	3	9	12
Total	14	25	39

Table 3. Comparison of public-private, accredited non-accredited schools

Comparison group	Mean	Standard deviation	p
Public	17.93	2.960	>0.05
Private	13.33	1.923	
Accredited	17.21	3.512	>0.05
Non-accredited	16.12	3.370	

Table 4. Comparison group results

Comparison group	Mean	Standard deviation	p
2012 scores	12.47	2.386	>0.05
2021 scores	13.27	2.520	

20 categories, which could be difficult to secure for newly established schools. The 14 schools are accredited and as expected 11 of the schools in 14, were established before 2012.[15] Exceptionally, only one school was a private school among these accredited 14 schools, and it was established after 2012. Even though time is a conceivable issue for the accreditation status, the tendency of private pharmacy schools to be involved in the accreditation process seems to lack currently.

Notably, the HEIs are in a competitive environment, where there are too many offers that could be suitable for them. Therefore, the attraction and retainment of students are significant issues in their financial status. Since the students are their customers certainly, they object to the market rules.[22] Thus, the private schools are searching for ways to increase their market presence in consideration of market mix. In this context, regarding 7P's of the market mix, promotion is knocking on the HEI's door. As an important channel of communication, websites are a unique tool to provide information and communicate directly with the candidates as they are the potential customers.[23] The total scores of public schools are higher than private ones, which is interesting considering funding options and marketing actions. Consequently, the accredited schools have higher scores. Yet, statistically there is no significant difference between accredited and non-accredited schools. As we consider the accreditation process, which is a questionable position for quality of the website of a pharmacy school, it is found that there is no significant difference between public and private schools.

Over the past decades or so, the changes in higher education have been dramatically affected by web technologies. With all kinds of information, such as paper-based documents or interactive sources, the unprecedented characteristics of websites make them the main way to present and access information.[24] Apart from these characteristics, the COVID-19 pandemic has affected our lives and usage of websites remarkably. Associated with e-learning programs and web-based education, which are accessible through websites, have secured their positions.[25] Although the development of websites has been progressive, it has not prevailed for pharmacy schools in Türkiye. We found that there is no statistical difference in websites of pharmacy schools, between 2012 and 2021 regarding their aspects.

To have websites as an indicator of quality and catch up with the era, the accreditation standards should include criteria for the web sites of the schools. Furthermore, the visibility of the websites of the faculties is another notable factor. In this context, to comply with some criteria that will increase the visibility will also be beneficial. Whereas internet has a countless number of pages, users prefer to use the search engines to reach each site precisely. Search engines are a kind of software, which collect information about websites, such as URL address, keywords or keywords groups defining the content, technical information and links that are provided on the website. Studies have shown that users tend to click on the first five results and ignore the remained. Considering user behavior, it is notable to use search engine optimization to move the website on the top of the search engine results.[26,27]

Study limitations

Number of pharmacy schools is increasing day-by-day, hence, it is impossible to include all schools in this study.

CONCLUSION

Websites, as the main communication tools in today's world, their importance for HEIs is indisputable. In this context, pharmacy schools as the HEIs should keep up to date with their official websites, considering the virtual world we have fell into with the developments in web technologies, besides the COVID-19 pandemic. Having websites as a category and the formation of standards in the accreditation process of pharmacy schools may increase communication with their students, apart from other stakeholders. Following the communication augmentation, it will be beneficial for both the brand image of public and private schools. Further studies and assessments on students' perspectives could be a milestone in the standardization process. Last but not the least, recognizing the importance of web-based communication tools will be a starting point to be prepared for worldwide crises such as pandemics.

Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.Y., B.S.Ş., L.Y., Design: S.Y., B.S.Ş., L.Y., Data Collection or Processing: S.Y., B.S.Ş., L.Y., Analysis or Interpretation: B.S.Ş., L.Y., Literature Search: S.Y., B.S.Ş., L.Y., Writing: L.Y., B.S.Ş., S.Y.

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ORIGINAL ARTICLE

Poloxamer and Chitosan-Based *In Situ* Gels Loaded with *Orthosiphon stamineus* Benth. Extracts Containing Rosmarinic Acid for the Treatment of Ocular Infections

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ABSTRACT

Objectives: *Orthosiphon stamineus* Benth. (OS) is a commonly used medicinal plant for curbing bacterial infections globally. This work aimed to fabricate poloxamer and chitosan-based *in situ* gels loaded with standardized aqueous-ethanolic OS leaf extracts and investigate their antimicrobial efficacy as a potential remedy against ocular infections.

Materials and Methods: *In situ* gels containing 0.5% w/v OS extract prepared using cold dispersion method were subjected to physicochemical characterization, including *in vitro*-release studies. Antimicrobial efficacy was tested against *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, and *Pseudomonas aeruginosa* using agar diffusion method.

Results: Thin layer chromatography and high performance liquid chromatography chromatograms confirmed the presence of rosmarinic acid (RA) and sinensitin in OS extracts with same retention factor (0.26 and 0.49) and retention times (12.2 and 20.7 min) against reference standards. A homogenous brown coloured *in situ* gel exhibited low viscosity as a solution and increased viscosity in gel form at ocular temperature. The optimized formulations, P7 (21% P407/4% P188), P8 (21% P407/5% P188) and F5 (1.5% chitosan and 45% β-glycerophosphate) exhibited ideal ocular pH (7.27-7.46), phase transition at ocular temperature (33-37˚C) and prolonged RA release up to 12 h. Formulation F5 showed an inhibition zone of 4.3 mm against *M. luteus*.

Conclusion: Among all, formulation F5 alone exhibited modest antimicrobial activity against *M. luteus*. OS extracts at 5% and 10% were most active against tested bacteria however, loading them into *in situ* gels resulted in sedimentation. Hence, isolation of RA from OS extract is suggested before loading into formulations for a better antimicrobial activity.

Key words: Chitosan, *in situ* gels, ocular infections, *Orthosiphon stamineus*, poloxamer

INTRODUCTION

Ocular infections such as conjunctivitis, keratitis, endophthalmitis, blepharitis, and orbital cellulitis if left untreated, damage the structures of the eye, which may possibly lead to visual impairments and blindness.[1,2] Conventional ocular dosage forms such as eye drop therapy require frequent administration, have limited duration of action and encounter rapid drainage from the eyes leading to poor ocular drug bioavailability (<5%).[3] To overcome these limitations, *in situ* gels can be a potential alternative to eye drops as they exist as free-

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flowing liquids before instillation into the eyes and transform into semi-solid gels upon exposure to ocular conditions such as pH, temperature or ionic concentration.[4] Although numerous *in situ* gelling systems have been developed, thermosensitive *in situ* gels are attracting popularity because of their swift response to a change in the temperature of surrounding environment. Thermosensitive *in situ* gels exist as a solutions at room temperature (25˚C) but transform at ocular temperature (33-37˚C) into gel.[5,6]

Several polymers have been employed in the development of *in situ* gelling systems intended for ocular delivery. However, being biocompatible, biodegradable, non-toxic, and bio-adhesive, chitosan is an ideal polymer.[3,7] Nevertheless, because of pH-dependent the activity of chitosan, it would not render a thermosensitive *in situ* gel.[8] However, complexation of chitosan with β-glycerophosphate (β-GP) that is a polyol salt, could yield a thermosensitive *in situ* gel system.[9,10] Apart from chitosan, poloxamers (Pluronics®) are also employed in the preparation of thermosensitive *in situ* gels. Poloxamers are tri-block copolymers that exhibit amphiphilic nature attributed to hydrophilic polyethylene oxide (PEO) attached to central hydrophobic polypropylene oxide (PPO).[11] Poloxamers are available in various grades owing to different weight ratios of PEO and PPO.[3] Among them, poloxamer 188 (P188) and poloxamer 407 (P407) grades are equally effective due to their safety in the ophthalmic region and clarity of their aqueous solution.[11] Hence, these grades were selected in this research to prepare thermosensitive *in situ* gels.

Despite the significant role of antibiotics and other antimicrobial agents for treating infectious diseases, side effects and emerging resistance have compromised their efficacy.[12,13] Hence, this situation has called for urgent action to turn the search lights on the development of naturally derived novel bioactive antimicrobial compounds from plants to fight against new and re-emerging infectious diseases with greater efficacy, lower toxicity and resistance. *Orthosiphon stamineus* Benth. (OS, Lamiaceae), also known as "Misai Kucing", is a popular plant in the Southeast Asian and tropical countries including Malaysia for curbing various ailments such as diabetes, inflammations, abdominal pain, oedema, and gout.[14] OS has been reported to be rich in phenolic bioactive compounds, namely rosmarinic acid (RA), sinensetin (SIN) and eupatorine, which present profoundly in the leaves of OS.[15] OS extract loaded into ethosomal formulation using sophorolipid as a surfactant has exhibited anti-angiogenic and anti-melanoma effects in albino mice, after topical application.[16] Hitherto, no published reports are available on the loading of OS extract into *in situ* gel formulation to evaluate the antimicrobial profile. Hence, this hypothesis-driven preliminary study was undertaken with objective of investigating the antimicrobial efficacy of OS extract-loaded thermosensitive *in situ* gelling systems against the selected pathogenic microorganisms responsible for various ocular infections.

MATERIALS AND METHODS

Experimental part

Materials

The dried leaves of OS were obtained from Ethno Resources (Malaysia). The standards of RA and SIN were purchased from Chemfaces (China). Chitosan, β-GP disodium salt, poloxamers and solvents including ethanol, methanol, acetonitrile, lactic acid, toluene, ethyl acetate, formic acid, and benzalkonium chloride (BAC) were purchased from Sigma-Aldrich, USA. Sodium chloride (NaCl) was purchased from Friendemann Schmidt, Australia. Mueller-Hinton agar was procured from Oxoid, Hampshire, UK. All reagents and chemicals were used as received.

Preparation of aqueous-ethanolic OS leaf extract

An aliquot (50 g) of dried OS leaves was blended (Waring 800S, USA) followed by maceration in 500 mL of ethanol-water (50:50) solvent (EWS) followed by filtration performed by a vacuum pump (Rocker 300, Malaysia). The evaporation of the filtrate was conducted using a rotary evaporator (Heidoph-36001270, Heidolph™, Germany) followed by lyophilization using a freeze dryer (Scanvac CoolSafe 9L, Denmark) for 48 h. The crude-dried extract was stored in a desiccator at ambient room temperature until further use.

Thin layer chromatography (TLC)

An extract of OS was dissolved in EWS to prepare standard solutions of 1, 5, and 10 mg/mL, whilst the reference standards were RA and SIN which were dissolved in methanol [high performance liquid chromatography (HPLC) grade] to prepare a solution of 1 mg/mL. The stationary phase was TLC plates (20 cm x 10 cm) pre-coated with silica gel 60 F254 (E. Merck, Germany), whereas the mobile phase comprised ethyl acetate:toluene:formic acid at a ratio of 7:3:0.1, respectively.[17] Following the air-drying of the TLC plate, images were taken under uv light at 254 nm and 366 nm. The band separation was observed followed by the measurement of the retention factor (R_f) values of the compounds using the formula as given below.[17]

$$Rf = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}} \qquad \text{equation 1}$$

Fingerprinting and quantification of aqueous-ethanolic OS extracts using HPLC

The test samples were prepared by dissolving 5 mg of freeze-dried OS extract in 1 mL EWS followed by dilution to 1 mg/mL. Standard solutions of RA and SIN at a strength of 0.5 mg/mL were prepared using methanol (HPLC grade) followed by serial dilutions to prepare a concentration range between 12.5 and 500 µg/mL. An injection volume of 20 µL was introduced into a C18 column (5 µm, 4.6 mm x 250 mm) in triplicate. The gradient mobile phase of 0.1% formic acid solution and acetonitrile (Table 1) was used at a flow rate of 1.5 mL/min.[18] The analysis was performed using HPLC (Agilent Technologies 1200, USA)

at a detection wavelength of 320 nm. The identification of RA and SIN in OS extracts was performed by comparing their R_ts with those of standards. Quantification was performed using the peak area of chromatograms obtained.

Preparation of OS extract-loaded poloxamer and chitosan-based in situ gels

A cold dispersion method was employed to prepare poloxamer-based *in situ* gels. In this method, accurately weighed poloxamer grades P188 and P407 were dissolved in phosphate-buffered saline (PBS, pH 7.4) using a magnetic stirrer at 300 rpm for 1 h followed by overnight refrigeration to allow complete hydration at 4˚C (Table 2). OS extract along with NaCl and BAC was incorporated into hydrated formulation with constant stirring until a homogenous solution was seen. Chitosan-based *in situ* gels were fabricated by adding chitosan polymer (1.5% w/v) in lactic acid (1.5% v/v) with constant stirring at 300 rpm to obtain a homogenous solution (Table 2). β-GP solutions (10-50% w/w) were formed using (PBS, pH 6.8) followed by overnight storage at 4˚C overnight to get a clear solution. OS extract along with NaCl and BAC was incorporated into β-GP solution followed by constant stirring until a homogenous solution was obtained. This solution was incorporated dropwise into chitosan solution with constant stirring until a homogenous solution was obtained. All prepared formulations were subjected to storage at 4˚C till required for use.

Table 1. Gradient elution system in separation of bioactive compounds present in OS extract

Time	0.1% Formic acid in water (%)	Acetonitrile (%)
0.01	95	5
25.00	50	50
26.00	20	80
35.01	95	5
45.00	95	5

OS: *Orthosiphon stamineus*

Physicochemical characterization

Physicochemical characterization of the preparation *in situ* gels was performed in terms of appearance, pH, sol-to-gel transition temperature, and viscosity.[3] The appearance of the *in situ* gels was observed against a background (black and white), while pH was determined using a pHmeter (EUTECH instrument, USA). To determine the temperature of sol-to-gel transition, solution form of *in situ* gel was transferred into an empty vial followed by immersing in a water bath adjusted initially at a temperature of 25˚C followed by a gradual increase up to 50˚C. The vial was overturned frequently to observe the gel formation and the temperature of sol-to-gel transition was recorded once the solution converted into a complete gel with no flow upon wobbling of the vial. The viscosity of the *in situ* gel formulations before and after gelation was determined using a rheometer (Anton Paar, Stockholm, Sweden) spindle type CC17 at a speed between10 and 100 rpmi with a shear rate of 0-100 mm/s. To determine the suitable sterilization technique for the sterility of prepared *in situ* gels (P7 and F5 formulations), two sterilization methods, namely sterilization using an autoclave at 121˚C for 15 min (Tomy SX-500, Japan) and sterilization using 0.20 µm sterile regenerated cellulose syringe filters (Sartorius, Germany) under aseptic conditions were employed.

Table 2. Composition of poloxamer and chitosan-based *in situ* gels loaded with OS extract

Poloxamer-based *in situ* gels containing OS extract								
Ingredients (% w/v)	P1	P2	P3	P4	P5	P6	P7	P8
OS extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Poloxamer 407	15	18	21	21	21	21	21	21
Poloxamer 188	-	-	-	1	2	3	4	5
Benzalkonium chloride	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Sodium chloride	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
PBS pH 7.4 qs ad	100	100	100	100	100	100	100	100
Chitosan-based *in situ* gels containing OS extract								
Ingredients (% w/v)	**F0**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	
OS extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Chitosan in 1.5% lactic acid	1.5	1.5	1.5	1.5	1.5	1.5	1.5	
β-glycerophosphate	0	10	20	30	40	45	50	
Sodium chloride	0.9	0.9	0.9	0.9	0.9	0.9	0.9	
Benzalkonium chloride	0.01	0.01	0.01	0.01	0.01	0.01	0.01	
PBS pH 6.8 qs ad	100	100	100	100	100	100	100	

OS: *Orthosiphon stamineus*, PBS: Phosphate-buffered saline

In vitro release of RA

The *in vitro* release of RA was performed using dialysis membrane technique reported in our earlier publications with suitable modifications.[3,19] The first ever drawn schematic representation of dialysis membrane method that illustrates the *in vitro* RA-release measurement procedure in a step-by-step manner is shown in Figure 1. A dialysis membrane with a molecular weight cut-off of 3500 Da (Fisher Scientific, New Hampshire, USA) was soaked in the dissolution medium of simulated tear fluid (STF, pH 6.8) overnight. About 1 mL formulation was transferred into the dialysis bag, which was subsequently placed into a beaker filled with a 50 mL STF, pH 6.8 and placed in a shaking water bath maintained at 37°C and 50 rpm. A fixed volume of 1 mL sample was taken at predetermined time intervals between 0.5 and 12 h followed by replacement with an equal amount of fresh STF to preserve the sink condition. RA released from *in situ* gels was analysed at a wavelength of 320 nm using ultraviolet (UV)-visible spectrophotometer (GENESYS™ 10, Thermo Scientific, USA). Percentage cumulative RA released *versus* time was plotted the drug release profile was characterized by estimating the time taken to release 50% of RA ($t_{50\%}$).[20]

Antimicrobial efficacy of OS extract loaded in situ gels

The antimicrobial efficacy of standardized aqueous-ethanolic OS leaf extracts loaded *in situ* gels against Gram-negative bacteria (*Escherichia coli* ATCC 10798 and *Pseudomonas aeruginosa* ATCC 9721) and Gram-positive bacteria (*Micrococcus luteus* ATCC 49732 and *Staphylococcus aureus* ATCC 6538) was determined using the agar well diffusion method. Autoclaved Mueller-Hinton agar (Oxoid, Hampshire, UK) was transferred into sterile petri dishes followed by inoculation using 100 µL bacterial broth culture (0.5 McFarland standard). Agar wells (6 mm in diameter) were prepared using the tip of a sterile pasteur pipette followed by filling with 70 µL tested OS extracts (0.5% to 10%) and OS extracts loaded *in situ* gel formulations, respectively. RA (1 mg/mL). Xepanicol®, 0.5% (chloramphenicol) and Ciplox®, 0.3% (ciprofloxacin) eye drops were employed as a positive control. The incubation of petri dishes was performed for 24 h at 37°C followed by the measurement of the resultant zones of inhibition (ZOI).

Statistical analysis

The results were evaluated statistically using one-way ANOVA followed by *post hoc* Tukey's test (GraphPad prism version 6.01, GraphPad software, La Jolla, California). The value $p<0.05$ was considered statistically significant difference.

RESULTS

TLC and HPLC analyzes of OS EWS extracts

OS EWS extract exhibited a percentage yield of 14.5% (w/w) on a dry weight basis. TLC chromatograms demonstrated that both OS extract and reference standards (RA and SIN) exhibited same Rf values at 0.26 and 0.49, respectively, when viewed under UV light at wavelengths of 254 nm (Figure 2a) and 366 nm (Figure 2b) confirming the presence of bioactive compounds RA and SIN. The HPLC chromatograms of reference standards (RA and SIN) and aqueous-ethanolic OS leaf extracts are shown in Figure 3. Both RA and SIN were eluted at the same retention times as of reference standards, RA (12.2 min) and SIN (20.7 min). A relatively higher content of RA (11.50 ± 0.23% w/w) was found in aqueous-ethanolic extract of OS leaves of 5 mg/mL compared to SIN (0.88 ± 0.02% w/w). However, SIN was unquantifiable in aqueous-ethanolic OS extract of 1 mg/mL, whereas the content of RA was measured as 11.37 ± 0.76% (w/w).

Physicochemical characterization of in situ gel formulations

Except for formulation P3 prepared using 21% P407, all *in situ*

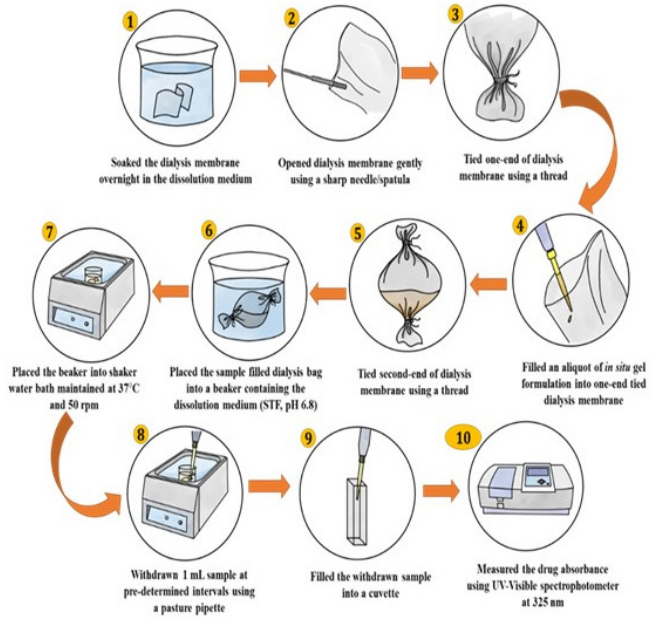

Figure 1. Schematic representation of *in vitro* release testing of RA from *in situ* gel formulations using dialysis membrane method

RA: Rosmarinic acid

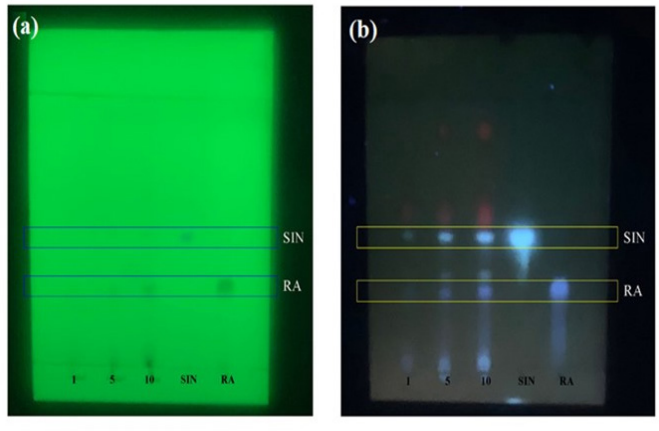

Figure 2. TLC chromatograms of the aqueous-ethanolic leaf extract of OS (1 mg/mL, 5 mg/mL and 10 mg/mL), and reference standards (RA and SIN) at 254 nm (a) and 366 nm (b)

TLC: Thin layer chromatography, RA: Rosmarinic acid, SIN: Sinensetin

gel formulations were found to be clear, homogenous and free flowing solution with a brown colour, suggesting easy instillation into the eyes at an ambient room temperature (Table 3). The pH of poloxamer based *in situ* gels was in a range between 7.21 and 7.32. The sol-to-gel phase transition temperature of *in situ* gel formulations is depicted in Figure 4. *In situ* gels comprising merely of P407 at 21% (w/v) content exhibited highest viscosity of 2440 mPas at room temperature. Nevertheless, the introduction of P188 (1-5%) at 21% P407 caused a gradual reduction in the viscosity from 166 to 116 mPas before gelation and increased viscosities from 337 to 5750 mPas after the gelation (Table 3). Chitosan-based formulations demonstrated a substantial reduction in the viscosity from 25.9 to 11.5 mPas before gelation and increased viscosity after gelation from 211

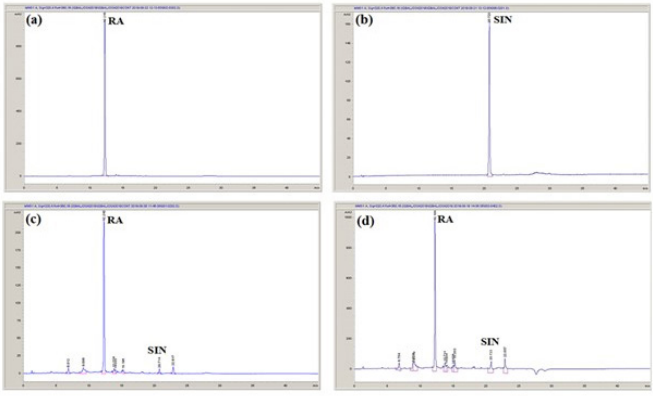

Figure 3. HPLC chromatograms of (a) standard RA at t_r: 12.215 min; (b) standard SIN at t_r: 20.720 min; (c) aqueous-ethanolic leaf extract of *OS* (1 mg/mL) showing peaks corresponding to RA (t_r: 12.243 min) and SIN (t_r: 20.714 min); (d) aqueous-ethanolic leaf extract of *OS* (5 mg/mL) showing peaks corresponding to RA (t_r: 12.188 min) and SIN (t_r: 20.723 min)

HPLC: High performance liquid chromatography, RA: Rosmarinic acid, SIN: Sinensetin, *OS: Orthosiphon stamineus*

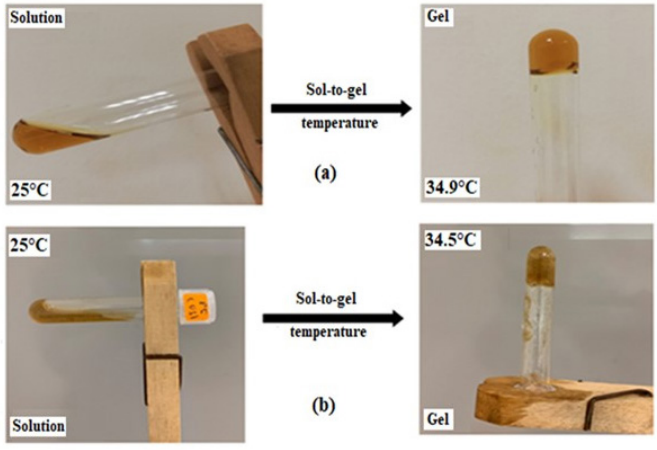

Figure 4. Depiction of sol-to-gel phase transition temperature of poloxamer-(a) and chitosan-(b) based *in situ* gels loaded with OS extract

OS: Orthosiphon stamineus

Table 3. Physicochemical characterisation of poloxamer and chitosan based *in situ* gels loaded with OS extract (mean ± SD, n: 3)

Formulation code	Appearance and clarity	pH	Sol-to-gel temperature (°C)	Viscosity (mPas)	
				Before gelation	After gelation
Poloxamer-based *in situ* gel formulations					
P1	Clear and free flowing	7.21 ± 0.01	No gelation until 50°C	27.30 ± 0.38	-
P2	Clear and free flowing	7.23 ± 0.01	No gelation until 50°C	58.01 ± 0.46	-
P3	Clear and viscous (gel)	7.29 ± 0.01	24.9 ± 0.06	2440.00 ± 17.52	-
P4	Clear and free flowing	7.32 ± 0.01	27.9 ± 0.10	166.18 ± 3.15	337.52 ± 4.16
P5	Clear and free flowing	7.30 ± 0.01	31.5 ± 0.21	150.37 ± 2.66	319.81 ± 2.43
P6	Clear and free flowing	7.22 ± 0.01	32.9 ± 0.36	138.25 ± 2.01	1280.16 ± 8.66
P7	Clear and free flowing	7.27 ± 0.01	33.6 ± 0.10	125.09 ± 2.45	3510.34 ± 11.02
P8	Clear and free flowing	7.30 ± 0.01	34.9 ± 0.12	116.01 ± 3.38	5750.47 ± 18.49
Chitosan-based *in situ* gel formulations					
F0	Clear and free flowing	4.56 ± 0.01	No gelation until 50°C	26.30 ± 0.77	-
F1	Clear and free flowing	7.09 ± 0.01	No gelation until 50°C	58.90 ± 1.23	-
F2	Clear and free flowing	7.28 ± 0.01	No gelation until 50°C	77.96 ± 1.84	-
F3	Clear and free flowing	7.41 ± 0.01	40.3 ± 1.27	25.90 ± 1.35	211.00 ± 4.81
F4	Clear and free flowing	7.37 ± 0.01	38.7 ± 0.85	14.75 ± 0.81	126.81 ± 3.42
F5	Clear and free flowing	7.46 ± 0.01	34.5 ± 0.07	12.37 ± 0.35	120.00 ± 6.19
F6	Clear and free flowing	7.47 ± 0.01	32.4 ± 0.71	11.50 ± 0.18	111.57 ± 5.05

SD: Standard deviation, OS: *Orthosiphon stamineus*

to 111 mPas with an increased β-GP concentration from 30 to 50% (F3-F6). The two sterilization methods employed did not affect the appearance, flow property, sol-to-gel temperature and viscosity results, however compared with autoclave sterilization, syringe filter sterilization demonstrated a small reduction of 0.1 unit in pH value. Additionally, the sterilized P7 and F5 *in situ* gel formulations exhibited increased RA content (47.67% and 45.13%) and reduced RA content (6.23% and 5.39%) following the sterilization using autoclave and filtration techniques, respectively, in comparison to the freshly prepared *in situ* gels.

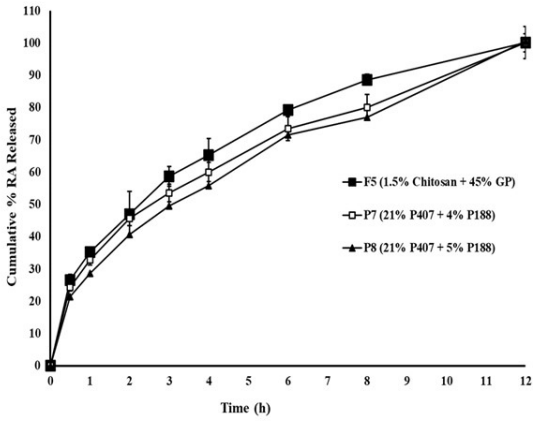

Figure 5. *In vitro* release profile of RA from chitosan (F5) and poloxamer (P7 and P8)-based *in situ* gel formulations (mean ± standard deviation, n: 3)
RA: Rosmarinic acid

In vitro release study of RA

Based on the physicochemical characterization especially sol-to-gel temperature findings, F5, P7, and P8 formulations were preferred for *in vitro* RA release and antimicrobial efficacy investigations. The *in vitro* release of RA from chitosan-β-GP and poloxamer-based *in situ* gel formulations are depicted in Figure 5. A substantial burst release of 35%, 32.81%, and 28.73% within the first 60 min and after that a gradual and complete RA release in a sustained manner for a period of 12 h was observed from F5, P7, and P8 formulations, respectively.

Antimicrobial efficacy of standardized aqueous-ethanolic OS leaf extracts loaded in situ gel formulations

Table 4 and Figure 6 show the ZOI of the tested samples against the Gram-positive and negative bacteria. Formulation F5 loaded with 0.5% (w/v) OS extract exhibited a modest antimicrobial efficacy against Gram-positive *M. luteus* (ZOI: 4.3 mm). However, it was, inactive against *S. aureus* and *P. aeruginosa*. To further test the antimicrobial efficacy of OS extract, the loading concentration of OS extract in P7 and P8 formulations was increased from 0.5% to 1% (w/v). However, both formulations were also found to be inactive against all tested bacteria (ZOI: 0.3 mm against *S. aureus*). Subsequent tests, which involved the use of OS leaf extracts at 0.5-10% (*i.e.*, E0.5-10%) yielded a concentration-dependent antimicrobial activity against all tested bacteria except *P. aeruginosa*. The extracts, especially E5% and E10%, were most active against *M. luteus* (highest ZOI: 16.0 mm), followed by *S. aureus* (highest ZOI: 9.3 mm) and *E. coli* (highest ZOI: 3.3 mm). As expected, reference products (*i.e.*, positive controls), chloramphenicol (Xepanicol®, 0.5%) and ciprofloxacin (Ciplox®, 0.3%) elicited very active

Table 4. ZOI of the tested samples against Gram-positive and negative bacteria

Treatments	ZOI (mm), mean ± SD (n: 3)			
	Gram-positive bacteria		Gram-negative bacteria	
	Micrococcus luteus	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*
Chloramphenicol (Xepanicol®; 0.5%)	42.3 ± 1.5[a]	26.0 ± 1.0[a]	29.7 ± 0.6[b]	24.7 ± 0.6[a]
Ciprofloxacin (Ciplox®; 0.3%)	32.7 ± 1.2[b]	26.3 ± 1.2[a]	33.3 ± 0.6[a]	20.3 ± 1.5[b]
Standard RA (1 mg/mL)	3.0 ± 1.0[d]	1.3 ± 0.6[e]	3.0 ± 0.0[c]	4.7 ± 0.6[c]
F5 (0.5%)	4.3 ± 2.1[d]	0.0 ± 0.0[e]	NT	0.0 ± 0.0[d]
P7 (0.5%)	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
P7 (1%)	0.0 ± 0.0[e]	0.3 ± 0.6[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
P8 (0.5%)	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
P8 (1%)	0.0 ± 0.0[e]	0.3 ± 0.6[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E0.5%	3.3 ± 0.6[d]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E1%	4.3 ± 0.6[d]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E3%	15.3 ± 0.6[c]	4.3 ± 0.6[d]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E5%	16.0 ± 1.0[c]	6.7 ± 0.6[c]	1.7 ± 0.6[d]	0.0 ± 0.0[d]
E10%	16.0 ± 1.0[c]	9.3 ± 0.6[b]	3.3 ± 0.6[c]	0.0 ± 0.0[d]

[a-e]Different letters represent significant differences ($p<0.05$) between treatments. NT: Not tested, ZOI: Zones of inhibition, SD: Standard deviation, RA: Rosmarinic acid

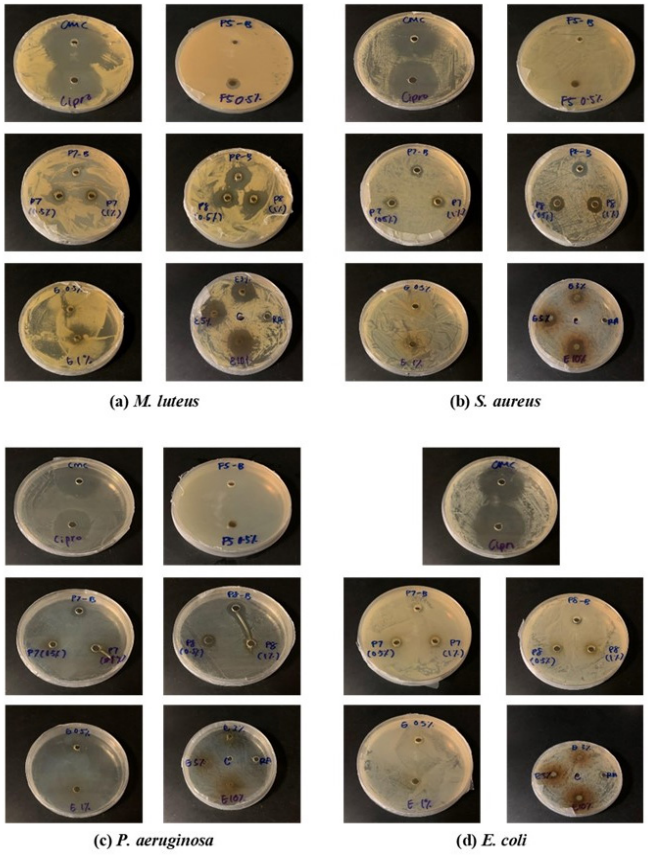

Figure 6. Representative images indicating ZOI of the tested samples against Gram-positive bacteria (a) *Micrococcus luteus*; (b) *Staphylococcus aureus* and Gram-negative bacteria (c) *Pseudomonas aeruginosa*; (d) *Escherichia coli*

ZOI: Zones of inhibition

antimicrobial efficacy against all tested bacteria (ZOI $\geq$ 20.3 mm). The standard RA, on the other hand, produced a modest antimicrobial activity, when tested against both Gram-positive and negative bacteria at 1 mg/mL (ZOI $\leq$4.7 mm).

DISCUSSION

The extraction of OS leaves was successfully with a percentage yield of approximately 14.5% (w/w) on a dry weight basis, which was consistent with an earlier reported study.[21] TLC analysis of OS extract exhibited robust band separation of the intended bioactive compounds, namely RA and SIN and these results are in agreement with a previous study.[17] Also, considerable amount of RA (11.5%, w/w) was quantified by HPLC analysis of OS extract at 5 mg/mL, which is primarily accountable for the antimicrobial activity of OS extract.[14] The use of water and ethanol binary mixture at 50:50 ratio as a solvent extract yielded a high content of phenolic compounds including RA.[22]

Apart from formulation F1 (0% β-GP), chitosan and β-GP based *in situ* gel formulations were homogenous, clear, and with pH values within a range of 6.8-7.4, ideal for the ophthalmic tissues depicting that *in situ* gels were non-irritating to eye.[23] All chitosan-based *in situ* gel formulations exhibited low viscosity at 25˚C pivotal for their existence in the solution form to ease the expulsion from the eye drop container.[3] Increasing the β-GP concentration from 10% to 50% (w/v) led to a further reduction in the viscosity (p<0.05), which could be ascribed to the attachment of the amine group of chitosan with the phosphate group of the β-GP.[24] The sol-to-gel transition temperature of these formulations dropped significantly to 32.4˚C from 38.7˚C upon increasing the β-GP concentrations from 30 to 50% (w/w). In the absence of β-GP, chitosan did not exhibit sol-to-gel phase transition (formulation F0) indicating the thermal insensitivity of chitosan to the surrounding temperature. Interestingly, only F5 (1.5% chitosan and 45% β-GP, w/v) transformed into gel at the temperature range between 33˚C and 37˚C that might be attributable to the enhancement in hydrogen bonding among chitosan chains upon a rise in temperature rise leading to gelation.[25] This gelation caused an enhancement in viscosity to 120 mPas from 12.3 mPas before gelation, which aided in increasing the reside time of formulation at pre-corneal surface.[19] Based on the ideal characteristics of *in situ* gels, formulation F5 was selected for further evaluation in terms of *in vitro* release and the antimicrobial activity.

Except for P3 (P407 at 21% w/v), poloxamer-based *in situ* gels were clear, homogenous, and free-slowing at 25˚C, whilst P3 formed viscous gels even at room temperature (Table 3). This was not surprising as it was reported that P407 would form a gel at an ambient room temperature at content above 20% (w/v) sol-to-gel and that the gelation phenomenon is reversible.[26,27] The pH of all poloxamer-based formulations was within a limit pivotal for ocular surface and suitable for ocular application without any irritation.[28] The viscosity of poloxamer-based formulations was significantly enhanced by increasing P407 concentration to 21% (w/v). Apart from P5, a significant increase in the viscosity of *in situ* gels from 337 to 5750 mPas after gelation was observed, when compared to before gelation at room temperature (p<0.05). These findings were in agreement with a previous report stating that the size and number of micelles within gel increase with a rise in the polymer concentration, which after that leads to enhanced cross-link between micelles by shortening the inter-micellar distance, resulting in the formation of three-dimensional gel structure with higher viscosity.[29] Among all, P7 and P8 formulations demonstrated the highest viscosities upon transition into a gel. An optimum *in situ* gel must exhibit sol-gel transition at a temperature above 25˚C; but below the temperature of eye surface (33-37˚C). Nonetheless, increasing the concentration of P407 from 18% to 21% (w/v), led to the incapability to undergoing gelation until 50˚C and formulation P3 was formed a gel-like structure at room temperature. This is attributed to the formation of additional micelles at P407 concentration above its critical micelle concentration, which reduced energy required for endothermic micellar crystallization and consequently sol-gel temperature was decreased.[30] Nonetheless, an addition of P188 at content between 1 and 5% (w/w) enhanced the sol-to-gel transition temperature for the subsequent formulations (P4-P9) to 34.9˚C from 27.9˚C. This is ascribable to the disruption in the formation of P407 micelles upon incorporation of hydrophilic

P188 leading to accumulation of excessive water in the proximity of hydrophobic PPO units of P407. This could have increased the required energy to initiate hydrophobic interactions among P407 micelles and consequently increased the temperature of sol-gel transition.[26] Only P7 and P8 formulations demonstrated optimum pH and phase transition at ocular temperature and, hence, were chosen for further studies.

Autoclave-sterilization dramatically increased the content of phenolic compound, RA, to almost double with respect to the freshly prepared and unsterilized *in situ* gel formulations. This could be ascribed to the heat employed during the autoclave process (121°C), which promoted the release of phenolic compounds due to breakdown of cell walls and cellular components. The dissociation of conjugated polyphenols such as tannins to simpler phenolic compounds due to the thermal process could have also contributed to the increased amount of RA.[31] The attained results are in similar agreement with the previously reported literature on the sterilization effect on the bioactive compounds present in the natural plant extracts.[32,33] The sterilization of *in situ* gel formulations by filtration technique demonstrated a small decrease in the RA content, which might be due to the sorption, where the substance is adsorbed onto the filter at the solid-liquid interface and removes the active constituent from the formulation.[34] Besides, the filtration of *in situ* gel formulations through 0.2 µm was found to be cumbersome due to the slightly viscous nature of the formulations because of the presence of poloxamer and chitosan polymeric content. Hence, this study confirms that autoclave sterilization is a suitable technique for the sterility of *in situ* gels as it did not exert any negative effect on the formulation characteristics and rather increased the content of RA, which could aid in further enhancing the antimicrobial efficacy of the prepared formulations.

The *in vitro* release studies of RA from the optimization *in situ* gel formulations demonstrated an initial burst release at first hour followed by a gradual and sustained release for 12 h, which is pivotal for the effective treatment of ocular infections. The $t_{50\%}$ of the formulations F5, P7, and P8 were found to be 2.26 h, 2.55 h, and 3.06 h, respectively. No statistically significant differences (p>0.05) between the release profiles of RA from F5, P7, and P8 in terms of $t_{50\%}$ values were found, suggesting a closely similar *in vitro*-release profiles.[20] The sustained release of RA from formulation F5 was attributed to chitosan polymer in the *in situ* gelling system capable of not only enhancing the viscosity of the formulation but also strong adhesion to the mucous layer of the precorneal area.[8] The sustained delivery of RA from poloxamer-based formulations might be attributed to enhanced rheological properties of formulations after gelation, which led the formation of tightly packed micellar structure, while initiating increased intermolecular interactions in the *in situ* gelling system.[35]

The chitosan-based *in situ* gel formulation (F5) loaded with 0.5% (w/v) OS extract exhibited modest antimicrobial activity against Gram-positive *M. luteus*. However, poloxamer-based *in situ* gel formulations, P7 and P8, loaded with either 0.5% (w/v) or 1% (w/v) OS extracts and, however, were inactive against all tested bacteria. Subsequently, the resultant antimicrobial activity of OS extract at concentrations ranging between 0.5%-10% (w/v) indicated a concentration-dependent antimicrobial activity against all tested bacteria except *P. aeruginosa*. The antimicrobial activity of OS is attributed primarily to the pro-oxidative properties of the RA.[36] Additionally, RA could also induce bacterial cell death by altering the charge and hydrophobicity of the bacterial membrane surface.[37] The present findings indicated OS extract, especially E5% and E10%, to be the most active against *M. luteus*. This agrees with previous findings documenting the partial to full effectiveness of OS extracts against Gram-positive bacteria, especially *M. luteus*, at a concentration above 3%.[38] The ineffectiveness of OS extract against Gram-negative bacteria, was also consistent with the previously reported findings.[39] This could be ascribed to an additional fortification for the microorganisms provided by extra outer membrane.[40] An attempt to incorporate 5-10% (w/v) OS extract into *in situ* gels resulted in dark-colored formulations with insoluble sediments. This could be due to the presence of an elevated content of lipophilic compounds in the OS extract.[41] Dark-coloured formulations could impair vision and mitigate the treatment adherence because clarity is a pivotal characteristic of any ocular formulation.[42] Nevertheless, the solubility of OS extract at relatively higher concentrations in the *in situ* gel formulation could be enhanced using various methods such as particle size reduction, inclusion of surfactants and complexation.[43,44] Moreover, purified RA isolated from OS extracts might also demonstrate promising antimicrobial activity.

Study limitations

The OS extract exhibited a moderate antimicrobial activity indicating concentration-dependent improvement against the antimicrobial activity especially against Gram-positive bacteria. Nonetheless, elevating the content of OS extract in the *in situ* gelling system resulted in dark-coloured sediments. This raises the need for further research to improve the clarity of *in situ* gel formulations loaded with OS extract at high concentrations. Further research is also recommended for isolation of RA from OS extract before incorporation into *in situ* gels for achieving a potential antimicrobial activity.

CONCLUSION

Standardized aqueous-ethanolic leaf extract of OS was obtained successfully using water: Ethanol binary mixture (50:50) as solvent *via* a maceration method. The presence of bioactive phenolic compounds, namely RA and SIN, in the OS extract was confirmed through TLC and HPLC methods. The optimized thermosensitive poloxamer and chitosan-based *in situ* gels loaded with OS extract, P7, P8, and F5 demonstrated a pH and sol-to-gel temperature suitable for ocular region. Additionally, release of RA was sustained up to a period of 12 h. Sterilization by autoclave was found to be a better technique than sterilization by filtration for achieving the sterility of *in situ* gel formulations. Formulation F5 loaded with 0.5% OS extract exhibited modest antimicrobial activity against *M.*

luteus, which appeared to be concentration dependent and indicated that increasing OS extract to 10% (w/v) may improve the antimicrobial activity, especially against Gram-positive bacteria. However, high concentrations of OS extract in the *in situ* gelling system-induced dark-coloured sediments. In conclusion, preliminary findings of this study could serve as a new source in race to find novel plant-derived antimicrobial compounds to fight against the new and re-emerging infections caused by antibiotic-resistant pathogens that is posing an alarming concern across the globe.

Ethics

Ethics Committee Approval: This study does not involve any animal or human subjects and hence ethics committee approval was not required.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: F.Z., R.S., Design: F.Z., R.S., K.R., Data Collection or Processing: N.Z.W., I.D.S., K.A., Analysis or Interpretation: F.Z., R.S., S.M.L., Literature Search: N.Z.W., I.D.S., Writing: F.Z., R.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Whole Genome Sequencing of Antibiotic Resistant Genes in Isolates from Surfaces in a Science Laboratory

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ABSTRACT

Objectives: Isolates obtained from laboratory surfaces were identified and characterized.

Materials and Methods: Ten consecutive isolates were obtained from 30 sample surfaces of a University Science Laboratory in Edo State Nigeria in May, 2021. Swabs of surfaces from the laboratory were obtained aseptically. The sample swabs were streaked on MacConkey, eosin methylene blue, mannitol salt, and nutrient agar plates, respectively, and incubated appropriately. Distinct colonies were randomly obtained from culture plates and characterized phenotypically. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was used to analyze four isolates (40%) obtained by selection criteria. Susceptibility testing using antibiotics was performed for the identified isolates by Kirby-Bauer method for 15 antibiotics. Isolate characterization and identification of resistance determinants were determined using whole genome sequencing (WGS).

Results: Microorganisms identified included *Leclercia adecarboxylata*, *Enterobacter hormaechei*, *Atlantibacter hermanii*, and *Stenotrophomonas maltophilia*. Three identified isolates were antibiotics-resistant and were investigated by WGS. Resistance genes were found in all (100%) of the resistant laboratory isolates. The resistance determinants included β-lactamase genes, aminoglycoside modifying enzymes, *qnr* genes, sulfonamide, tetracycline, and trimethoprim resistance genes, respectively. Two isolates carried *ESBL* genes and $bla_{CTX\text{-}M\text{-}15}$ was detected.

Conclusion: Our study displays the dissemination of antibiotic resistance among isolates obtained from surface of a University Science Laboratory. To the best of our knowledge, we have reported the first genomic characterization of resistance to antibiotics in isolates obtained from surfaces of a University Science Laboratory in Nigeria.

Key words: Whole genome sequencing, antibiotic resistance, science laboratory surfaces

INTRODUCTION

Microorganisms are ubiquitous. They have been detected in several areas of the environment. Air, water, soil, and fluid from animals are carrier/vehicles of microorganisms. The quality of air is usually affected by the presence of microorganisms, which include bacteria, fungi, and viruses and people breathe in on average 14 m^3 of air *per* day.[1] Poor air quality, especially contaminated with microorganisms, can lead to severe health challenges for humans. Microorganisms are transmitted through other routes, which include contaminated food and food products, droplet contact by sneezing, coughing or contacting with contaminated surfaces or soil. The mechanisms how microorganisms attach to animate and inanimate things have been previously reported.[2] Biofilms may be found on a wide variety of surfaces. Physical forces determine how microorganisms are transmitted and attached to surfaces. Once bacteria get attached to surfaces, they start to divide, resulting in biofilms, which cause the complex structure of natural sediments.[3] The microorganisms may either be active

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and reproduce immediately or remain inactive on surfaces for long periods, making it difficult to identify the contamination source.[4] Antibiotic resistance is a serious health challenge not only among human pathogens, but also in isolates found in other habitats. Many resistant pathogenic bacteria and commensals are found in different hosts or in the environment at large with the potential of causing infections that are usually difficult to treat.[5] *Brucella* species, *Shigella* species, *Salmonella* species, *Mycobacterium tuberculosis*, and *Neisseria meningitidis* have been reported as the most common microorganisms causing laboratory-acquired infections (LAI). Infections because of the hepatitis virus, human immunodeficiency virus, and fungal infections caused by dimorphic fungi have also been commonly reported.[6] Laboratory-acquired and nosocomial infections pose an important challenge globally and the characterization of microorganisms causing such infections is important as it provides possible therapeutic solutions for some LAI. It is important to characterize microorganisms causing LAIs to devise procedures to prevent subsequent outbreaks.[7] This study aimed to identify isolates, revealing resistance to antibiotics in isolates from surfaces at Pharmaceutical Microbiology Laboratory, Igbinedion University Okada, and characterizing the resistance mechanisms using whole genome sequencing (WGS).

MATERIALS AND METHODS

Materials

Collection of samples

Thirty samples were obtained aseptically from the surfaces of work benches, tables, fridges, sinks, equipment, windows, and doors in pharmaceutical microbiology laboratory using sterile cotton swab sticks. One sample was obtained *per* surface. Sterile cotton swab sticks were soaked in sterile peptone water before sampling.

Media preparation and sterilization

Four culture media, *e.g.* eosine methylene blue agar, mannitol salt agar, Macconkey agar, and nutrient agar, were used in this study. All culture media were prepared and sterilized based on manufacturer's instructions.

Isolation and identification

Ten consecutive isolates were obtained in May, 2021 from 30 sample surfaces of work benches, tables, fridges, sinks, equipment, windows, and doors in the pharmaceutical microbiology laboratory of Igbinedion University Okada in Edo State, Nigeria. Samples were aseptically collected and immediately inoculated on agar plates. Inoculated plates were incubated at 37˚C for 24 h. Distinct colonies formed were randomly obtained from culture plates. Pure cultures were obtained afterwards on agar slants maintained at 4˚C in the refrigerator throughout the study. Identification of isolates was carried out using standard microbiological techniques.[8] Identities of randomly selected four isolates were subsequently confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonik GmbH, Bremen, Germany) analysis.

Antimicrobial susceptibility tests

The Kirby-Bauer susceptibility testing technique (Bauer et al.)[9] was performed and results were analyzed using European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria.[10] The isolates were tested using 15 antibiotics: ampicillin, meropenem, ertapenem, ceftazidime, cefotaxime, amoxicillin/clavulanic acid, cefoxitin, cefepime, cefpodoxime, tigecycline, ciprofloxacin, amikacin, piperacillin/tazobactam, cefuroxime, and gentamicin (Oxoid, Basingstoke Hampshire, UK).

Whole genome sequencing

WGS was carried out for four randomly selected isolates, whose identities were confirmed by MALDI-TOF mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) analysis. Genomic DNA (gDNA) extraction was carried out using the MagAttract HMW DNA extraction kit (Qiagen, Hilden, Germany). Quantification of gDNA was performed on a Qubit® 2.0 fluorometer using the dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 0.2 ng/μL based on the manufacturer recommendations (Illumina sample preparation guide, Illumina Inc, San Diego, CA, USA). Preparation of fragment libraries of the bacterial genomes was carried out using the Illumina Nextera XT DNA library preparation kit (Illumina Inc, San Diego, CA, USA). A DNA fragment library preparation was carried out using 1 ng of gDNA (Illumina sample preparation guide). Paired end sequencing using a read length of 2 x 300 bp on an Illumina Miseq (Miseq ver. 3.0, Illumina Inc) was performed using Miseq reagent kit v3 containing the reagent cartridge and flow cell. Pooled libraries were loaded on the reagent cartridge. Samples were sequenced to obtain a minimum average coverage of 100 fold based Illumina's recommended standard protocols.

Table 1. Genome assembly statistics of the recovered isolates

Isolate	Isolation source	Genome size	Genome coverage	N_{50} (bp)	Number of contigs	Accession number
Atlantibacter hermannii	Laboratory sink	4.7	28	175,641	114	JAJNEI000000000
Stenotrophomonas maltophilia	Laboratory bench	4.6	24	38,623	220	JAJNEH000000000
Leclercia adecarboxylata	Laboratory bench	4.9	51	142,618	158	JAJNEK000000000
Enterobacter hormaechei	Laboratory sink	4.7	85	244,270	88	JAJNEJ000000000

Raw reads (FASTQ files) were trimmed at their 5' and 3' ends until an average base quality of 30 was reached in a window of 20 bases, and assembly was performed using Velvet version 1.1.04 Zerbino[11], using optimized k-mer size and coverage cut values based on the average length of contigs with >1.000 bp. Species identification *via* MALDI-TOF MS was confirmed using ribosomal multilocus sequence typing (rMLST) (https://pubmlst.org/species-id). Identification of antimicrobial resistance genes (ARGs) was carried out using the comprehensive antibiotic resistance database-resistance gene identifier (RGI).[12] ARGs were identified on the basis of a minimum cut-off of 98% nucleotide identity for perfect or strict hits predicted by RGI. Sequences were analyzed for their plasmid replicon types using PlasmidFinder and their MLST using MLST 1.8 software, both available from the Center for Genomic Epidemiology.[13,14] No statistical analysis method was used to analyzing results.

Nucleotide sequence accession numbers

This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession numbers JAJNEH000000000-JAJNEK000000000. The version described in this paper is version JAJNEH010000000-JAJNEK010000000. Table 1 shows the genome assembly statistics of the recovered isolates.

Table 2. Identity of the isolates

Bacterial isolates	Identities
510509	*Atlantibacter hermannii*
510510	*Stenotrophomonas maltophilia*
510507	*Leclercia adecarboxylata*
510508	*Enterobacter hormaechei*

RESULTS AND DISCUSSION

Species identification using MALDI-TOF-MS and ribosomal MLST assigned 4 isolates to 4 different species (Table 2). Identified laboratory isolates include *Atlantibacter hermanii, Stenotrophomonas maltophilia, Enterobacter hormaechei,* and *Leclercia adecarboxylata* (Table 2). Out of 4 randomly selected identified isolates, 3 isolates revealed antibiotic resistance and were further analyzed by WGS (Table 3). No information about antibiotics is obtained in the EUCAST table for *S. maltophilia.* The laboratory isolates were ampicillin-resistant (100%), cefotaxime (67%), amoxiclav (100%), cefepime (67%), ceftazidime (67%), cefpodoxime (67%), cefuroxime (67%), ciprofloxacin (67%), gentamicin (67%), and tigecycline (67%). Two isolates were positive in the phenotypic testing of ESBLs and had *ESBL* gene $bla_{CTX\text{-}M\text{-}15}$ detected (Table 3).

WGS revealed that 3 out of 4 identified isolates harbored more than one resistance gene. Resistance genes were detected in *S. maltophilia*, but did not pass the minimum cut-off 98% nucleotide identity for perfect or strict hits predicted by RGI. The resistance determinants in the isolates included β-lactamase genes, $bla_{TEM\text{-}1}$, $bla_{ACT\text{-}24}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$; aminoglycoside modifying enzymes, *aac (3)-IId, aph(6)-Id, aph3")-Ib, aac(6')-Ib-cr4*; *qnr* gene, *qnr*B1; sulfonamide resistance gene, *sul2*; tetracycline resistance

Table 3. Antibiotic susceptibility test results of the isolates

Resistance testing *via* disk diffusion

ID no	Source	Identity	R-I						R-II							ESBL
			CTX 5-cefotaxime	FEP 30-cefepime	AMP 10-ampicillin	AMC 30-amoxicillin/ clavulanic acid	TZP 36-piperazillin/ tazobactam	CXM 30-cefuroxime	MEM 10-meropenem	ETP 10-ertapenem	CN 10-gentamicin	AK 30-amikacin	CIP 5-ciproflaxin	TGC 15-tigecyclin	FOX 30-cefoxitin
510507	Laboratory	*Leclercia adecarboxylata*	S	S	R	R	S	I	S	S	S	S	S	S	S
510508	Laboratory	*Enterobacter hormaechei*	R	R	R	R	S	R	S	R	S	S	R	R	R
510509	Laboratory	*Atlantibacter hermannii*	R	R	R	R	S	R	S	S	R	S	R	S	S
510510	Laboratory	*Stenotrophomonas maltophilia*													

Table 3. Continued

Resistance testing *via* disk diffusion

ID no	Source	Identity	ACI										XAMA				
			CPD 10-cefpodoxime	CAZ 30-ceftazidime	FEP 30-cefepime	CTX 5-cefotaxime	AMC 30-amoxicillin/ clavulanic acid	TZP 110-piperacillin/ tazobaactam	MEM 10-meropenem	TOB 10-torabmycin	AK 30-amikacin	SXT 25-trimethoprim/ sulfamethoxazole	CIP 5-ciprofloxacin	LEV 5-levofloxacin	MH 30-minocyclin	STX 25-trimethoprim/ sylfamethoxazole	CIP 5-ciprofloxacin
510507	Laboratory	*Leclercia adecarboxylata*	S	S	S	S	R										
510508	Laboratory	*Enterobacter hormaechei*	R	R	R	R	R										
510509	Laboratory	*Atlantibacter hermannii*	R	R	R	R	R										
510510	Laboratory	*Stenotrophomonas maltophilia*												No info	No info	I	No info

R: Resistant, I: Intermediate, S: Sensitive, ?: (-) no breakpoints, IE: Insufficient evidence that the organism or group is a good target for therapy with the agent, No info: No information about AB in EUCAST table for *Stenotrophomonas maltophilia*

gene, *tet*(D); phenicol resistance gene, *catII* and trimethoprim resistance gene, *dfrA14*. Other resistance determinants, which included the regulatory systems modulating antibiotic efflux CRP, antibiotic target alteration gene EF-Tu were also detected in the antibiotic resistant isolates. Table 4 shows the characteristics of the antibiotic resistant laboratory isolates. *E. hormaechei* isolated was of the sequence type ST 78. *A. hermanii, S. maltophilia,* and *L. adecarboxylata* had previously unknown sequence types (Table 4). Plasmids from the incompatibility group detected among the isolates were predominantly of Inc F and Col family types (Table 4).

Microbial contamination in a laboratory varies in different laboratories based on their geographical location and measures used to control infection, which poses an important challenge. Few reports have been made on this issue, especially in developing countries like Nigeria, which is really a drawback. Most of the previous studies were based on the phenotypic characterization of microbial isolates from surfaces in the laboratory. In a previous Nigerian study by Isola and Olatunji[15], bacterial isolates obtained from laboratory surfaces were characterized and identified. The results showed that the most frequent microorganisms from laboratory surfaces were *Bacilli*. Others included *Salmonella typhae* and *Staphylococcus aureus*. Another previous study identified *Staphylococcus epidermis* and aerobic spore bearers, *i.e. Bacillus subtilis,* a common microorganism contaminating working areas in a microbiology laboratory.[16] Strains identified were possible pathogens and could cause LAIs. In another study by Veena Kumari et al.[17], laboratory surface samples were assessed for microbial contaminants. Out of the 60 surface samples assessed, coagulase- negative *Staphylococci* were the most frequent contaminant, followed by Gram-positive bacilli (*Corynebacterium* spp.). In our study, isolates obtained were on a relatively small scale compared to previous studies. Significantly, no Gram-positive isolate was identified among the isolates obtained in this study. All isolates were Gram-negative bacteria. More than one resistance determinant was found on the draft genome sequences of the resistant isolates, which showed that they are potential pathogens that may cause LAIs.

Resistance mediated by $bla_{CTX\text{-}M\text{-}15}$ has been reported globally, including in Nigeria.[18-20] Recently, the first report on $bla_{CTX\text{-}M\text{-}15}$ in clinical isolates of *Providencia* spp., *Citrobacter freundii*, and *Atlantibacter hermannii* isolated from humans in Nigeria was published.[21] To the best of our knowledge, we report the first genomic characterization of resistance to antibiotics in isolates obtained from surfaces of a University Science Laboratory in Nigeria. This suggests the circulation of the gene $bla_{CTX\text{-}M\text{-}15}$ in a different setting. Significantly, resistance genes were detected in *L. adecarboxylata* observed to be sensitive to most of the antibiotics in the susceptibility testing. This shows the importance of studying antibiotic resistance not using phenotypic methods only but further genotypic and molecular characterization techniques like WGS.

Enterobacter cloacae ST66, ST78, ST108, and ST114 strains are known to be extended spectrum cephalosporin-resistant spread internationally as high-risk clones.[22] *E. hormaechei* ST78 isolate detected in this study belonged to the high-risk clone known particularly for the nosocomial spread carbapenemases and ESBLs.[22] Jesumirhewe et al.[21], in a recent report, detected *E. hormaechei* ST78 isolates in a Nigerian Hospital. *E. hormaechei* ST78 identified in this study suggests the circulation of this high-risk lineage in a different setting. The detection of the predominant plasmid replicon types (Inc F and Col) among the resistant isolates in this study displayed their importance in the transmission of antibiotic resistance. A recent report indicated that the IncF plasmid type is the most prevalent among human ESBL Enterobacteriaceae isolates in Nigeria.[21]

CONCLUSION

Microorganisms are ubiquitous including the laboratory environment. Techniques for reducing contamination should be employed, which include the use of soaps to clean laboratory surfaces, pre- and post- treatment of hands in disinfectant before conducting any experiment in the laboratory, the use of protective clothing, when working in the laboratory. Laboratory coats must be strictly used in the laboratory and unworn outside the laboratory. Measures should be aimed at eliminating/ significantly reducing these microorganisms from laboratory

Table 4. Details of the resistant isolates

Isolates	Sequence type	Plasmid replicon type	Antibiotic resistance genes/determinants detected
Enterobacter hormaechei	78	Col(pHAD28), Col3M	MD-regulatory system modulating antibiotic efflux CRP, antibiotic target alteration gene Ef-Tu ,BL- $bla_{ACT\text{-}24,}$ $bla_{CTX\text{-}M\text{-}15,}$
Atlantibacter hermanii	Unknown	IncFIB(pECLA), IncFII(pECLA)	*A-aac*(3)-*lld*, *aph*(6)-*ld*, *aac*(6')-*lb-cr*4, P-*catII, T-dfrA14,* MD-regulatory system modulating antibiotic efflux CRP,TE- tet(D), S-sul2, BL-$bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$ FQ-*QnrB1*
Leclercia adecarboxylata	Unknown	Col(pHAD28), IncFII(pCTU2)	*A-aph*(6)-*ld*, *aph*(3")-*lb*, T-*dfrA14,* MD-regulatory system modulating antibiotic efflux CRP,S-*sul*2, BL-$bla_{TEM\text{-}1}$
Stenotrophomonas maltophilia	Unknown	No plasmid replicon type	No resistance gene detected

A: Aminoglycosides, BL: *Beta*-lactams, MD: Multi-drug, P: Phenicol, T: Trimethoprim, TE: Tetracycline, S: Sulphonamide, FQ: Fluoroquinolones

using proper procedures. Frequent assessments of surfaces of the laboratory should be carried out not using phenotypic methods; only but molecular methods to identify and explore the genetic mechanisms of resistance to antibiotics in isolates, which is important to understand the dissemination of resistant isolates. Further molecular studies must characterize the prevailing clonal lineages and plasmids that harbor resistance mediating genes in the isolates. Frequent assessments would assist laboratories in either avoiding or eliminating most microbial contaminants found in the laboratory.

Ethics

Ethics Committee Approval: Not required for the study.

Informed Consent: Not required for the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: C.J., Design: C.J., Data Collection or Processing: C.J., A.O.A., Analysis or Interpretation: C.J., W.R., Literature Search: C.J., A.O.A., Writing: C.J.

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ORIGINAL ARTICLE

Risk Perception and Acceptability of the COVID-19 Vaccine in Nigeria

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ABSTRACT

Objectives: Vaccines are anticipated to control the ongoing coronavirus disease-2019 (COVID-19) pandemic, however, their acceptance is critical for the desired benefit. This study assessed risk perceptions of COVID-19, acceptability of its vaccine and socio-demographic associations of its acceptability in Nigeria.

Materials and Methods: A cross-sectional web-based study was conducted among 420 participants in Nigeria's six geopolitical regions, using a three-part questionnaire. The questionnaire link was distributed *via* snowball method to consenting participants through online platforms. Study outcome measures were acceptance of COVID-19 vaccine, and risk perception of COVID-19 by study participants. Descriptive and inferential statistics were performed using Microsoft Excel and SPSS version 24. p values ≤0.05 were considered statistically significant.

Results: A total of 410 respondents participated in the study and high-risk perception of severe acute respiratory syndrome-coronavirus-2 infection (COVID-19) was seen in 127 (66.1%) respondents. Vaccine acceptance was high in 233 (56.8%) respondents and was significantly associated with geo-political region (p=0.028). A moderate positive relationship (r: 0.3) was found between risk perception and acceptability of COVID-19 vaccine and the correlation was statistically significant (p=0.000).

Conclusion: High-risk perception of COVID-19 was found in over half of the respondents, and COVID-19 vaccine acceptance rate was a little more than 50%. However, the study noted regional association with vaccine acceptance among study participants. Therefore, strategic and targeted messaging on vaccine acceptance should be prioritized by stakeholders, to ensure successful vaccine implementation.

Key words: COVID-19, acceptability of vaccine, Nigeria, risk perception, infection

INTRODUCTION

The coronavirus disease-2019 (COVID-19) pandemic has been a major public health issue since its initial report in Wuhan, China.[1] Its associated negative effects on the economy and human socialization have impacted the quality of life and psycho-social health of individuals.[2] Originating from a novel coronavirus, severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2),[1] specific drug treatment has remained a challenge, although several drugs have been associated with improved outcomes of the disease.[3,4] Therefore, wide availability and acceptance of safe and effective vaccines against SARS-CoV-2 have become a major public health priority.

Several types of potential COVID-19 vaccines are currently being developed, with a good number of them at the clinical trial phase, and some already rolled out.[5,6] Some of these include, inactivated viral vaccines, non-replicating viral vector vaccine, protein subunit vaccine, replicating viral vector vaccine, ribonucleic acid based vaccine, virus-like particle, among others.[7] Major concerns of COVID-19 vaccines are efficacy and adverse reactions, especially in vulnerable groups of the populations.[5] Vaccine is potentially sufficient to confer herd immunity in communities and a subsequent control of the pandemic, but this requires high immunization coverage.[8] A rate of 55% to 82% has been estimated for SARS-CoV-2 herd immunity threshold.[9] Therefore, for effective control with COVID-19 through vaccination, it is critical to ensure the readiness of the population and acceptance of the vaccine by a large proportion of the population. Risk perception of the

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disease could also appreciably determine the acceptability of vaccine.[10]

About 160,000 confirmed cases of COVID-19 have been recorded in Nigeria as of April 3rd, 2021, since its first confirmed case on February 27th, 2020.[11] Although COVID-19 remains an ongoing pandemic with significant morbidity and mortality, low mortality and high recovery rates have been recorded in Nigeria.[11] Irrespective of the highly infectious nature of SARS-CoV-2, only a few persons appear aware of the possibility of contracting the disease in a Nigerian population with a good number regarding the disease to be exaggerated.[12] Denial and misplaced "religious zealotry" are also perceived to be negatively associated with COVID-19-related behavior in Nigeria.[13] Vaccination is critical to the prevention and control of infectious diseases, and previous studies have reported varying rates of vaccine acceptance.[14,15] Low rates was observed in Nigeria during poliomyelitis vaccination,[16] and this prevented the early eradication of wild polio in Nigeria. Meanwhile, vaccine hesitancy is a potential public health threat which is associated with the behavioral attributes of a people.[17]

It is evident that acceptance of COVID-19 vaccine is a major public health need for the effective control of the disease. Lower risk perceptions of COVID-19 may influence the potential willingness to accept it. This study assessed the risk perception of COVID-19 and acceptability of COVID-19 vaccine among the Nigerian population. Other studied points herein were associations between respondents' socio-demographic characteristics and vaccine acceptability. It is hoped that findings from this study will equip public health professionals and policymakers with insights and relevant information that can be used for optimal COVID-19 vaccine rollout.

MATERIALS AND METHODS

Study design and study setting

A cross-sectional web-based study was conducted from November 20th, 2020 to December 28th, 2020 to ascertain the risk perception of COVID-19 and the acceptance of the vaccine in Nigeria. Nigeria is located in West Africa with over 250 ethnic groups and is divided into six geopolitical zones and the Federal Capital Territory. Each geo-political region is made up of 6 states, except the northwest and southeast regions, which consist of 7 and 5 states, respectively. It is the most populous black country with a population of 200 millions.[18] The country had an average life expectancy of 54 years as of 2018,[19] and had been previously faced with several disease outbreaks.

Study population

Using vaccine acceptance of 50% and a margin of error of 5% (95% CI), we calculated a sample size of 385 individuals[20] for the study, but 10% was added to account for potential non-responses or attrition, and the study was then conducted among 420 participants.

The study included both male and female Nigerians, who resided in Nigeria and had access to internet facilities, and those who belonged to online groups. It however, excluded persons, who did not give informed consent for the study and persons, who were less than 19 years of age. State of residence was included among the measured variables, to exclude non-Nigerian residents.

Survey instrument

The questionnaire included questions that explored the respondents' risk perceptions of COVID-19 and their willingness to accept the vaccine.

A three-part questionnaire, which required about 3 min to complete, was designed using Google forms. The constructs measured by the questionnaire were the respondents' risk perception of COVID-19 and the acceptability of COVID-19 vaccine. Responses were required for all questions in the Google form. Section A of the questionnaire had 7 questions that obtained information on respondents' basic socio-demographic characteristics. Section B consisted of 5 questions and elicited information on respondents' risk perception of COVID-19. Section C was composed of 5 questions that assessed respondents' acceptability of a COVID-19 vaccine. The questions were anchored on a 5-point Likert scale that ranged from strongly agree to strongly disagree. "SARS-CoV-2 infection" was not used in the questionnaire, "COVID-19" was rather used to facilitate comprehension.

The questionnaire was validated by expert evaluations and preliminary tests. Three public health experts, who are colleagues of the authors and with more than 10 years of practice, assessed their suitability by face and content validity. The preliminary testing was conducted among 20 adults, who were randomly selected within the six geopolitical regions of Nigeria. Three adults comprising one male and 2 females were sampled from each region except the North-West region, where 5 adults were sampled following a larger number of states compared to others. The selected respondents comprised persons with tertiary education, less than tertiary education, and no formal education. This enabled the verification of proper understanding of the questions and modifications were made to questions that were deemed to be ambiguous. Modifications included restructuring of such questions to suite the understanding of respondents. Only questions one and two, under risk perception of SARS-CoV-2 needed such modification. The reliability of the survey tool was also assessed using Cronbach's alpha.

Data collection and study outcome measures

The internet links were distributed to consenting participants through online platforms. Snowball approach was adopted in the distribution of the internet links *via* social media platforms and the survey was closed after 6 weeks of data collection (November 20th, 2020 to December 28th, 2020). Reminders were sent to participants at intervals. The outcome measures for the study were acceptance rates of COVID-19 vaccine by the study participants and risk perception of COVID-19.

Ethical statement

Ethics approval was obtained from the Ethical Unit of the Kwara State Ministry of Health, and had the reference number MOH/

KS/EU/777/455. Informed consent was obtained from the study participants before the study. The first part of the online survey instrument had a clear statement to show that participation in the study was completely voluntary. As also stated in the first part of the survey, consent for study participation was implied by clicking on the link and submitting the completed form. Forms were completed and submitted anonymously, and confidentiality of the participants' information was ensured during and after the study.

Statistical analysis

Online survey data were downloaded onto Microsoft Excel spreadsheet for data cleaning, validation, and descriptive analysis. Determination of the survey sample characteristics was performed using descriptive statistics (frequencies, percentages). The assessment of perceived risk and vaccine acceptability, which both had a 5 point Likert scale, was interpreted as the following: "Strongly disagree," "disagree" and "undecided": 0, while "strongly agree" and "agree" for each question on risk perception and potential vaccine acceptability were scored 1, and the scores were added together. Weighted analyses of COVID-19 risk perception and COVID-19 vaccine acceptability were performed. Weighted scores were totaled over 5, and higher scores (≥3) denoted "high risk perception" and "vaccine acceptance" (pro-vaccination), respectively. Low scores (≤2) represented "low risk perception" and "vaccine unacceptance" (anti-vaccination).

Data were imported to SPSS version 24, where Pearson's chi-square analysis was conducted to determine associations between socio-demographics and vaccine acceptability. Pearson's correlation coefficient test was used to determine the relationship between risk perception and acceptability of COVID-19 vaccine. $p \leq 0.05$ was considered statistically significant.

RESULTS

A total of 420 respondents participated in the survey, but 10 were excluded as the participants were not resident in Nigeria. The Cronbach's alpha showed a reliability of 0.82.

Socio-demographic characteristics of the respondents are shown in Table 1. Majority of the study participants were male 241 (58.8%), aged between 26-35 years old 175 (42.7%), single 240 (58.5%), employed 301 (73.4%), had tertiary education 371 (90.5%) and from the North-Central Region of the country 133 (32.4%).

Details of respondents' risk perception of SARS-CoV-2 infection are summarized in Table 2. Majority agreed that COVID-19 is a severe disease 358 (87.3%), associated with stigma 284 (69.3%) and will affect many Nigerians 247 (60.2%). Meanwhile, less than half 165 (40.2%) perceived themselves to be susceptible to SARS-CoV-2 infection (COVID-19) and over half 225 (55.1%), perceived that their close relatives may get infected with SARS-CoV-2. Respondents' total weighted risk perception of SARS-CoV-2 infection was 127 (66.1%). This implied that 66.1% of the study participants had a high risk perception of COVID-19.

Table 1. Socio-demographic characteristics of respondents

Variables	Frequency (n: 410)	Percentage (%)
Gender		
Male	241	58.8
Female	169	41.2
Age		
18-25 years	117	28.5
26-35 years	175	42.7
36-45 years	90	22.0
46-55 years	18	4.4
>55 years	8	2.0
N/A	2	0.5
Marital status		
Married	167	40.7
Single	240	58.5
Widowed	1	0.2
Divorced	1	0.2
Separated	1	0.2
Employment status		
Employed	301	73.4
Unemployed	3	0.7
Student	86	21.0
Retiree	3	0.7
N/A	17	4.2
Highest educational qualification		
None	1	0.24
Primary	0	0.0
Secondary	34	8.3
Tertiary	371	90.5
Informal	1	0.2
N/A	3	0.7
Geo-political zones		
North-East	15	3.7
North-West	16	3.9
North-Central	133	32.4
South-East	38	9.3
South-West	89	21.7
South-South	114	27.8
N/A	5	1.2

Table 3 shows acceptability of the COVID-19 vaccine by respondents, where the total weighted acceptance of the vaccine was 233 (56.8%), therefore almost half 177 (43.2%) would not accept it. Majority would accept COVID-19 vaccine, if substantial information accompanied it 260 (63.4%), if they were recommended by their healthcare provider 283 (69.3%), and if it was provided at no financial cost 239 (58.3%). Conversely, less than half 173 (42.2%) would accept it irrespective of its novelty, and only about one third (1/3) of the population 134 (32.7%) will readily accept the vaccine regardless of its potential adverse effects.

Details of the socio-demographic associations of COVID-19 vaccine acceptability are given in Table 4. Acceptability of the vaccine was not significantly associated with gender (p=0.846), age (p=0.073), marital status (p=0.105), employment status (p=0.293), and educational qualification (p=0.556) but was associated with geo-political region (p=0.028). Over half of the males 136 (56.4%) and females 97 (57.4%) would accept the COVID-19 vaccine in Nigeria. Meanwhile, more than half of the persons between 36-45 years old 49 (54.4%) and persons above 55 years old 5 (62.5%), would not accept the vaccine. Similarly, about half 83 (49.7%), of the married respondents would not accept the COVID-19 vaccine, while a majority of 147 (61.3%) of the single respondents would accept it. Findings from the study showed that the region with potentially highest acceptance rate was the North-East 11 (73.3%) and the region with the least acceptance rate was the North-West 6 (37.5%). Over half of the persons from the South-East 22 (57.9%), South-West 49 (55.1%), North-Central 85 (63.9%), and almost half of the persons in the South-South 55 (48.2%), would accept the vaccine.

The result of the Pearson correlation coefficient test showed a moderate positive (correlation r: 0.3) between risk perception and acceptability of COVID-19 vaccine among the study participants. The correlation was statistically significant (p=0.000).

DISCUSSION

A high risk perception of COVID-19 was observed in the majority of the respondents. The impact of an infectious disease may depend on perceptions about the disease. Hence, risk perception of COVID-19 is expected to enhance the uptake of precautionary behaviors including vaccine acceptance. Meanwhile, a weak correlation was found between COVID-19 risk perception and practice of preventive measures in Nigeria.[12] Therefore, following the high infectivity, and associated morbidity and mortality of SARS-CoV-2, improving its risk perception by health education measures is essential. In an Iranian and a Nigerian study, socio-demographic factors were reported to be determinants of COVID-19 risk perception,[21,22] which re-enforces the need for targeted messaging by stakeholders.

Findings from this study indicate that almost half of the study participants will not likely accept COVID-19 vaccine, which is in congruence with previous studies in Nigeria.[23,24] This level of the vaccine acceptance may not be sufficient for COVID-19 herd

Table 2. Risk perception of SARS-CoV-2 infection (COVID-19) among respondents

Variables	Risk code (strongly agree and agree: 1, not sure, disagree and strongly disagree: 0)	Frequency (n: 410)	Percentage (%)
COVID-19 is a severe disease	1	358	87.3
	0	52	12.7
COVID-19 prevents regular associations	1	284	69.3
	0	124	30.2
	No response	2	0.5
I may likely get COVID-19 in the course of the pandemic	1	165	40.2
	0	244	59.5
	No response	1	0.2
There is a chance that my close relative may contract COVID-19	1	226	55.1
	0	181	44.2
	No response	3	0.7
COVID-19 will affect many Nigerians	1	247	60.2
	0	161	39.3
	No response	2	0.5
Total weighted risk perception	High risk	271	66.1
	Low risk	139	33.9

Weighted risk perception (high risk ≥3, low risk ≤2), SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2, COVID-19: Coronavirus disease-2019

Table 3. Acceptability of COVID-19 vaccine among respondents

Variables	Acceptability (strongly agree and agree: 1, not sure, disagree and strongly disagree: 0)	Frequency (n: 410)	Percentage (%)
If I am offered a COVID-19 vaccine with substantial information on the vaccine, I will accept it	1	260	63.4
	0	144	34.1
	No response	6	1.5
I will accept to take COVID-19 vaccine if my healthcare provider recommends it	1	284	69.3
	0	123	30.0
	No response	3	0.7
If the vaccine is provided at no financial cost, I will accept it	1	239	58.3
	0	169	41.2
	No response	2	0.5
Despite the fact that COVID-19 vaccine is new, I will accept it once it is made available	1	173	42.2
	0	234	57.1
	No response	3	0.7
I will accept COVID-19 vaccine regardless of my fears of potential adverse effects	1	134	32.7
	0	273	66.6
	No response	3	0.7
Total weighted acceptability of COVID-19 vaccine	Pro-vaccination	233	56.8
	anti-vaccination	177	43.2

Weighted acceptability (pro-vaccination ≥3, anti-vaccination ≤2), COVID-19: Coronavirus disease-2019

immunity. COVID-19 vaccine acceptance in the United States of America had a higher acceptance rate than ours,[25] and high-risk perception of the disease was associated with vaccine acceptance.[26] Perception of risks of COVID-19 is expected to enhance the uptake of precautionary behaviours[27] including vaccine acceptance. Therefore, the difference in findings may be associated with varying levels of risk perceptions among the populace, and the overall COVID-19 associated morbidities and mortalities in the two countries. Nigeria has recorded only about one hundred and sixty thousand confirmed cases, majority of whom had been discharged from the treatment centres, with very few deaths.[11] Previous studies observed that potential acceptance of the vaccine varied among countries, with China having the highest acceptance rates.[14,15] Meanwhile, available studies indicate presence of antibodies and other immune responses, and high effectiveness of COVID-19 vaccines against SARS-CoV-2 infection and COVID-19 related illness and death.[28,29]

Our study suggests that majority of persons may be cautious of the potential adverse effects of COVID-19 vaccine. This may be a major limiting factor to its acceptance, although positive beliefs about COVID-19 vaccine have been previously reported among the Nigerian population.[30] Similarly, almost half of the persons, who would accept the vaccine in China also considered confirmation of vaccine safety as a necessity for receiving a dose of it.[15] China's high vaccination rate has been attributed to several implemented strategies that are technical and non-technical, involving adequate local production of vaccines, free vaccination and trust in vaccine effectiveness, among others.[31] These strategies may also help in promoting vaccination in other settings. Meanwhile, considering that healthcare providers may have a positive influence on the vaccine acceptance through their recommendations, as seen in our study, it is pertinent to seek their total commitment regarding information on potential adverse reactions of the vaccine. This may build trust in the public and enhance vaccine acceptance.

In this study, the acceptance of COVID-19 vaccine was not significantly associated with gender, age, marital status, employment status, and educational qualification, but was associated with geo-political region. Similarly, a recent study in Nigeria did not observe associations between age/gender and COVID-19 vaccine acceptance.[24] In contrast, it was reported that sex and marital status could enhance the likelihood of COVID-19 vaccine acceptance in China.[15] Significant differences in socio-demographic associations with COVID-19 vaccine acceptance were also observed in the United States.[25] In our study, vaccine acceptance was majorly noted to be associated with geographic regions, this suggests the need for targeted educational interventions in the regions, with effective COVID-19 vaccine-acceptance detailing, *via* various media. Because of varying

Table 4. Associations between vaccine acceptability and respondents socio-demographic characteristics

Variables	Total (n: 410)	Anti-vaccination n (%)	Pro-vaccination n (%)	X^2	*p* value	Phi	Cramer's V
Gender							
Male	241	105 (43.6%)	136 (56.4%)	0.038	0.846	0.010	0.010
Female	169	72 (42.6%)	97 (57.4%)				
Age							
18-25 years	117	42 (35.9%)	75 (64.1%)	10.086	0.073	0.157	0.157
26-35 years	175	73 (41.7%)	102 (58.3%)				
36-45 years	90	49 (54.4%)	41 (45.6%)				
46-55 years	18	8 (44.4%)	10 (55.6%)				
>55 years	8	5 (62.5%)	3 (37.5%)				
N/A	2	0 (0.0)	2 (100%)				
Marital status							
Married	167	83 (49.7%)	84 (50.3%)	7.65	0.105	0.137	0.137
Single	240	93 (38.8%)	147 (61.3%)				
Widowed	1	1 (100%)	0 (0.0)				
Divorced	1	0 (0.0)	1 (100%)				
Separated	1	0 (0.0)	1 (100%)				
Employment status							
Employed	301	137 (45.5%)	164 (54.5%)	4.948	0.293	0.110	0.110
Unemployed	3	1 (33.3%)	2 (66.7%)				
Student	86	33 (38.4%)	53 (61.6%)				
Retiree	3	2 (66.7%)	1 (33.3%)				
N/A	17	4 (23.5%)	13 (76.5%)				
Educational qualification							
None	1	0 (0.0)	1 (100%)	3.012	0.556	0.080	0.086
Primary	0	0 (0.0)	0 (0.0)				
Secondary	34	16 (47.1%)	18 (52.9%)				
Tertiary	371	158 (42.6%)	213 (57.4%)				
Informal	1	1 (100%)	0 (0.0)				
N/A	3	2 (66.7%)	1 (33.3%)				
Geo-political zones							
North-East	15	4 (26.7%)	11 (73.3%)	**14.173**	**0.028***	**0.186**	**0.186**
North-West	16	10 (62.5%)	6 (37.5%)				
North-Central	133	48 (36.1%)	85 (63.9%)				
South-East	38	16 (42.1%)	22 (57.9%)				
South-West	89	40 (44.9%)	49 (55.1%)				
South-South	114	59 (51.8%)	55 (48.2%)				
N/A	5	0 (0.0)	5 (100%)				

*Statistically significant

educational levels in the regions, this significant association is expected. However, without adequate interventions, this could impair the formation of herd immunity threshold for SARS-CoV-2[9] in the country. Basic interventions may include building trust in the vaccine, through consistent thoughtful and targeted public health information.

Findings from the study showed a positive relationship between risk perception and acceptability of COVID-19 vaccine among study participants. Vaccine acceptability moderately increased with an increase in risk perception. This corroborates with findings from China.[32] Therefore, an increase in public enlightenment on effects of COVID-19 is likely to enhance acceptance of the vaccine. This suggests targeted interventions that will improve knowledge and risk perception of COVID-19 among persons of various demographics, for improved vaccine acceptance.

Study limitations

The study was faced with some limitations, among which may be selection bias, following the online method of data collection that may have excluded persons in rural communities with no internet facilities and older adults, who may not be friendly with social media applications. Therefore, this may have over-estimated the rate of acceptability of the vaccine, thereby limiting the generalization of our findings. Likewise, a low response rate recorded in the north-east and north-west regions may limit the generalization of the findings in these regions. Lastly, merging the "undecided" group with the "disagree" and "strongly disagree" groups may have caused losses of some statistical outcomes during dichotomization of COVID-19 vaccine acceptance. However, the study buttressed the probability of vaccine acceptance from high-risk perception of COVID-19, across socio-demographic variables.

CONCLUSION

A high risk perception of COVID-19 was found in over half of the respondents. Similarly, COVID-19 vaccine acceptance rate was found to be a little more than 50%. However, the study noted regional associations with vaccine acceptance among the study participants. Therefore, more targeted and strategic educational interventions are necessary to improve risk perception and acceptance of COVID-19 vaccine in order to break the disease transmission dynamics.

Ethics

Ethics Committee Approval: Ethics approval was obtained from the Ethical Unit of the Kwara State Ministry of Health and had the reference number MOH/KS/EU/777/455.

Informed Consent: Informed consent was obtained from the study participants before the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: C.O.I., O.H.E., Design: C.O.I., O.H.E., C.N.A., Data Collection or Processing: C.O.I., O.H.E., Analysis or Interpretation: C.O.I., O.H.E., Literature Search: C.O.I., Writing: C.O.I., O.H.E.

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ORIGINAL ARTICLE

Pharmacist's Knowledge and Behaviors Toward Pharmacovigilance and Adverse Drug Reactions Reporting Process in Türkiye

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ABSTRACT

Objectives: Adverse drug reactions (ADRs) increase patient-related morbidity and mortality. Additionally, it is an important public health problem associated with prolonged hospital stay and increasing economic burden. Pharmacovigilance is central to reducing ADRs, so the development and growth of this science is critical to effective and safe clinical practice. The aim of the study was to evaluate the knowledge and behaviors of pharmacists toward pharmacovigilance and spontaneous ADR notifications in Türkiye.

Materials and Methods: The online questionnaire method was used with the pharmacists, whose prior consent was obtained to participate in the study. The survey was uploaded onto Google form. The survey link was distributed electronically to the eligible participants *via* social media channels. The knowledge of pharmacovigilance practice, ADR reporting compliance rates, reasons for not reporting ADR, and perceptions of the Turkish pharmacists on pharmacovigilance practice were evaluated.

Results: Four hundred six pharmacists (45%) agreed to participate in the study, 81.8% of whose correctly defined correctly defined the term pharmacovigilance. 91.6% knew the name of the Turkish Pharmacovigilance Center. Clinical and hospital pharmacists were found to have a more adequate knowledge than community pharmacists ($p<0.05$). 18.7% of pharmacists stated that they had previously reported ADRs. Most of the pharmacists stated that the most important reason for not reporting ADRs was not knowing how and where spontaneous reporting should be done, a single spontaneous reporting would not make a difference and the report would generate extra work.

Conclusion: These results showed that Turkish pharmacists had adequate knowledge about the concept of pharmacovigilance and the spontaneous ADR reporting system. However, they had little experience in reporting. Training programs should continue to increase the knowledge and reporting experience of pharmacists about the reporting process and requirements.

Key words: Adverse drug reaction reporting system, pharmacists, pharmacovigilance, knowledge, behavior

INTRODUCTION

Adverse drug reactions (ADRs) increase patient-related morbidity and mortality. Additionally, it is an important public health problem associated with prolonged hospital stay and increasing economic burden.[1,2] Pharmacovigilance is central to reducing ADRs, so the development and growth of this science is critical to effective and safe clinical practice. The World Health Organization defines pharmacovigilance as "science and activities related to the detection, evaluation, understanding, and prevention of side effects or other possible drug-related problems".[3]

Healthcare professionals are central to providing a robust pharmacovigilance system. Consumers are more likely to report ADRs to their physicians or pharmacists than to the pharmaceutical industry.[4,5] All health system sectors should be included in the reporting process.[6] Pharmaceutical care involves assessing these risks on a patient-by-patient basis by "identifying and solving (or preventing)" drug therapy problems.

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Therefore, pharmacists play an important role in safe drug use and should be aware of this.[7] Unfortunately, the rate of self-reporting of ADRs by healthcare professionals around the world is extremely low, as it is not a mandatory requirement in most countries.[4,5] After the thalidomide disaster in the 1960s, many countries developed their national pharmacovigilance centers.[8] "Türkiye Adverse Drug Effects Monitoring and Evaluation Center" is Türkiye's first national pharmacovigilance center, which was established in 1985. In 2005, its name was changed to "Turkish Pharmacovigilance Center" (TUFAM). According to the regulations, all healthcare professionals have to report a serious and unexpected ADR to TUFAM within 15 days, either directly by post, fax, or e-mail, or through the pharmacovigilance contact point in the healthcare institutions where they work.[9] Many studies from other countries show the role and attitudes of pharmacists in ADR reporting.[5,10-13] There are a limited number of studies evaluating the knowledge and behavior of pharmacists regarding pharmacovigilance and spontaneous ADR reporting in Türkiye.[14] This study evaluates the knowledge and behaviors of pharmacists regarding pharmacovigilance systems and spontaneous ADR reporting in Türkiye.

MATERIALS AND METHODS

Study design and settings

This cross-sectional study was conducted between April 10th, and May 10th, 2021. The online questionnaire method was used with the pharmacists, whose prior consent was obtained to participate in the study. The survey was uploaded onto Google form. The first page of the survey contains information about the research subject. There was an option to either consent to or refuse participation in the survey at the end of this page. The individuals, who chose to participate, were allowed to complete the survey. The survey link was distributed electronically to the eligible participants *via* social media channels (Instagram, WhatsApp, and Facebook). Ethical approval for this study was obtained from the Gazi University Ethics Committee (approval number and date 2021-445/06.04.2021).

Sampling technique and sample size

An online questionnaire was applied to randomly selected community pharmacists, hospital pharmacists, and clinical pharmacists in Türkiye. Academic pharmacists working at the university and non-active pharmacists were excluded from the study.

According to the data of the Turkish Pharmacists Association, there are 37.442 pharmacists in Türkiye. The online Raosoft sample size calculator (http://www.raosoft.com/samplesize.html) estimated the sample size of a minimum of 381 pharmacists to provide a 95% confidence level with a 5% margin of error, assuming 50% of pharmacists express good knowledge.

Data collection

The questionnaire items and question selection were based on previous research and interviews with senior pharmacists on this subject. A draft questionnaire was designed to be subjected to tests and examinations by community pharmacists (n: 10) and hospital pharmacists (n: 10). The survey questions were then adjusted according to the qualitative feedback provided by the respondents and the results of the internal validity measurement. Cronbach alpha score was 0.6. There were 19 questions in the study. The first 5 questions were based on the demographic information of the participants, while 6-11 questions were about knowledge and 12-18 of the questions were about behavior.

Statistical analysis

Statistical analyses of the main survey data were performed using IBM SPSS (version 24.0) with significance levels set at $p \leq 0.05$. Demographic variables and responses given to knowledge and behavior questions were analyzed using descriptive statistics. Descriptive analyses were used to represent the results as percentages and frequencies. For knowledge questions, correct answers were scored as 1, and wrong answers were scored as 0. Six questions were calculated as 6 points and corresponded to 100%. A score of more than 80% was accepted as adequate knowledge, while a total score ≤80% was classified as inadequate knowledge. Scoring was not done for behavior questions. Association between patients' socio-demographic characteristics and ADR knowledge was also assessed using the Pearson chi-square test.

RESULTS

The questionnaires were sent to 900 pharmacists, whereas 406 pharmacists (45%) agreed to participate. 73.4% of the respondents were women and 51.3% of the respondents were between the ages 22-29. Community pharmacists constituted 65% of the respondents, while 21.4% were hospital pharmacists and 13.5% were clinical pharmacists. The respondents rated at 54.2% had a working period as a pharmacist in less than 5 years and 17.2% of them had a postgraduate degree. Socio-demographic characteristics of pharmacists are summarized in Table 1.

Table 1. Demographic characteristics of pharmacists (n: 406)

Variables	n (%)
Gender	
Female	298 (73.4)
Male	108 (26.6)
Age, ranges	
22-29	210 (51.7)
30-44	82 (20.2)
45-59	90 (22.2)
>60	24 (5.9)
Work place	
Community pharmacist	264 (65)
Hospital pharmacist	87 (21.4)
Clinical pharmacist	55 (13.5)
Experience as a pharmacist	
<5 years	220 (54.2)
≥5 years	186 (45.8)
Postgraduate degree (MSc, PhD)	
Yes	70 (17.2)
No	336 (82.8)

Knowledge

Table 2 demonstrates the responses to questions related to knowledge. The respondents rated at 81.8% defined the term pharmacovigilance correctly, whereas 46.3% of the respondents correctly answered the location of the world pharmacovigilance center. 91.6% knew the name of Türkiye's National Pharmacovigilance Center. 79.8% of the respondents answered correctly, who could report ADRs and 83.3% knew which ADRs could be reported correctly. 70.4% of the respondents knew how to report an ADR. Table 3 shows the association with socio-demographic characteristics of patients and pharmacovigilance awareness as well as ADR knowledge and reporting of previously experienced ADRs. 57.6% of the respondents had sufficient knowledge of ADRs. The relationship of ADR information with age, gender, duration of the study, and postgraduate degree was not found to be statistically significant ($p>0.05$). The relationship between the pharmacist's work area (clinical pharmacist or hospital pharmacist or community pharmacist) and the previous reporting of ADRs was significant ($p<0.05$). Clinical and hospital pharmacists were found to have better knowledge levels than community pharmacists.

Table 2. Knowledge of the pharmacists concerning pharmacovigilance and reporting ADRs

Questions	n (%)
What is pharmacovigilance?	
Adverse drug reaction reporting	52 (12.8)
Detection, recognition, evaluation, and prevention of adverse drug reactions*	332 (81.8)
The science of evaluating the benefit/risk profile of a medicinal product	16 (3.9)
Do not know	6 (1.5)
Where is the World Pharmacovigilance Center located?	
United States of America	174 (42.9)
France	30 (7.4)
United Kingdom	14 (3.4)
Sweeden*	188 (46.3)
Which institution is responsible for adverse reaction reporting and monitoring in Türkiye?	
TUFAM*	372 (91.6)
The Regional Board of Pharmacists	-
Turkish Pharmacists Association	20 (4.9)
Do not know	14 (3.4)
Who can spontaneously report adverse drug reactions?	
Doctor	12 (3)
Pharmacist	70 (17.2)
Dentist	-
Nurse	-
All of above*	324 (79.8)
What types of adverse drug reactions are expected to be reported?	
Serious and unexpected	58 (14.3)
Not serious	-
Expected adverse reactions	2 (0.5)
All adverse reactions regardless of seriousness and expectedness*	338 (83.3)
Do not know	8 (2)
Do you know how to report adverse drug reactions?	
Yes*	286 (70.4)
No	120 (29.6)

*True answer, ADRs: Adverse drug reactions, TUFAM: Turkish Pharmacovigilance Center

Behaviors

Table 4 shows that pharmacists' behavior toward reporting ADRs. 40.4% of pharmacists stated that they did not experience ADRs in their patients at all, 39.9% experienced them once a year, 15.8% once a month, and 3.9% once a week. Previously, 76 pharmacists (18.7%) were found in the ADR notification.

55.2% were serious, 31.5% were unexpected, and 5% were rare type ADRs. 99.5% of the respondents stated that it was important to report ADRs. The relationship between the pharmacist's work area (clinical pharmacist, hospital pharmacist or community pharmacist) and the previous reporting of ADRs was significant ($p<0.05$). 35.6% of hospital pharmacists, 23.6% of clinical pharmacists, and 12.1% of community pharmacists, who participated in our study, declared that they had previously reported ADRs.

Some of the respondents (36.9%) stated that the most important reason for not reporting ADRs was that they did not know how and to where spontaneous reporting should be done. 19.2% of them stated that a single spontaneous reporting would not make a difference and 18.7% stated that the report would generate extra work (Table 5). Among the factors that encourage reporting ADRs are; the reaction was serious (31.5%), the reaction was unexpected (22.7%), the training of healthcare professionals (19.2%), and the reporting process was practical and easy (17.7%) (Table 6).

DISCUSSION

The results of our study revealed that although Turkish pharmacists had sufficient pharmacovigilance and theoretical knowledge about ADRs, they showed a low rate of ADR reporting. 81.8% of the respondents correctly defined the term pharmacovigilance. This was a fairly high rate. In the study by Kopciuch et al.[11] 73% of the respondents, 69.5% of the respondents in the study by Su et al.[15], and 81.9% of the respondents in the study by Li et al.[5] defined the term pharmacovigilance correctly. In the study by Suyagh et al.[16], 25.5% of the participants correctly defined the term pharmacovigilance. In our study, 91.6% of respondents correctly knew the institution reporting national ADRs in Türkiye, while nearly half of the respondents in the study by Kopciuch et al.[11] knew this correctly in the study by Suyagh et al.[16] 76% of the respondents declared that they did not know where to find the necessary forms for ADR reporting and 60% of the respondents did not know the national pharmacovigilance center in the study by Vigneshwaran et al.[17] Toklu and Uysal[14] evaluated the knowledge and attitudes of pharmacy pharmacists in Türkiye regarding ADRs in 2008.

Table 3. Association of pharmacists' demografic characteristics with pharmacovigilance awareness, knowledge of adverse drug reaction reporting

Variables	ADR knowledge	
	Adequate (score >80%)	Inadequate (score <80%)
Gender	$p>0.05$	
Female	176 (75.2)	122 (70.9)
Male	58 (24.8)	50 (29.1)
Age (years)	$p>0.05$	
22-29	132 (56.4)	78 (45.3)
30-44	52 (22.2)	30 (17.4)
45-59	38 (16.2)	52 (30.2)
>60	12 (5.1)	12 (7)
Work place	$p=0.01$	
Community pharmacist	128 (54.7)	136 (79.1)
Hospital pharmacist	63 (26.9)	24 (14)
Clinical pharmacist	43 (18.4)	12 (7)
Experience as a pharmacist	$p>0.05$	
<5 years	136 (58.1)	84 (48.8)
≥5 years	98 (41.9)	88 (51.2)
Postgraduate degree (MSc, PhD)	$p>0.05$	
Yes	44 (18.8)	26 (15.1)
No	190 (81.2)	146 (84.9)

ADR: Adverse drug reaction

Table 4. Pharmacists behaviors towards reporting ADRs

	n (%)
How often do you see ADRs in patient?	
Once a week	16 (3.9)
Once a month	64 (15.8)
Once a year	162 (39.9)
Never	164 (40.4)
Have you ever previously reported adverse drug reactions?	
Yes	76 (18.7)
No	330 (81.3)
If you have reported, what type of adverse drug reaction was the most common?[a]	
Serious	42 (55.2)
Rare	10 (13.1)
Unexpected	24 (31.5)
Do you think adverse reaction reporting is important and necessary?	
Yes	404 (99.5)
No	2 (0.5)
If your answer is yes, what is the most important reason?[b]	
To increase patient safety	-
To indicate relatively safe drugs	190 (47)
To determine the incidence of adverse reactions	74 (18.3)
To identify new adverse reactions	140 (34.6)

[a]n=76, [b]n=404

Table 5. The most important factor that may discourage pharmacists from reporting adverse drug reactions

	n (%)
Lack of time to complete reports	48 (11.8)
Concern that the report will generate extra work	76 (18.7)
Not paying a fee for notification	2 (0.5)
Concern about submitting an inappropriate report	18 (4.4)
Not knowing how and where spontaneous reporting should be done	150 (36.9)
Incomplete medical history of the patient	34 (8.4)
The idea that a single spontaneous reporting cannot make a difference	78 (19.2)

Table 6. The most important factor that encourages pharmacists to report adverse drug reactions

	n (%)
Reaction to a new drug	16 (3.9)
Unexpected reaction	92 (22.7)
Serious reaction	128 (31.5)
Payment asset for instant reporting	8 (2)
Requiring spontaneous notification	12 (3)
Practical and easy spontaneous reporting process	72 (17.7)
Training of healthcare professionals	78 (19.2)

In the study, 17% of pharmacists correctly defined what pharmacovigilance was. This low rate may have resulted from the question being an open-ended question and it was only 3 years ago that the regulation on the pharmacovigilance system (Regulation on Monitoring and Evaluation of the Safety of Medicinal Products for Human Use) came into force. 87% of pharmacists did not know where to get ADRs reporting forms.[14] The results of our study showed that the level of knowledge on pharmacovigilance and ADRs reporting increased significantly in Türkiye. This was because pharmacovigilance courses are given in more places during the bachelor's degree. In our study, 40.4% of pharmacists stated that they had never seen ADRs in their patients, which was a high rate. In the study by Suyagh et al.[16], 8% of the patients never reported to their pharmacist, when ADRs developed. This might be because patients in Türkiye did not consult pharmacists, when ADRs developed. In the patient information leaflet of some medicines in Türkiye, there is the phrase "consult your doctor when you have an unexpected side effect". This statement may have prevented patients who experienced ADR the pharmacist from consulting, when ADRs develop. Pharmacists should also be added to this statement in the patient information leaflet. Although 99.5% of the pharmacists thought that ADR reporting was important, 18.7% of pharmacists previously reported ADRs. This ratio was also supported by other scientific reports that concluded that the rates of ADRs reported by pharmacists in various countries ranged from 14.6% to 38%.[5,11,15,17-19] But this ratio was 7% in the study by Toklu and Uysal.[14] This situation demonstrated that the ADR reporting rate has increased in Türkiye.

CONCLUSION

These results showed that Turkish pharmacists had sufficient knowledge about the concept of pharmacovigilance and the spontaneous ADR reporting system. However, they had little experience in ADRs reporting. Training programs should be ongoing to enhance the role of pharmacists, their knowledge of the reporting process, and requirements and the reporting experience.

Ethics

Ethics Committee Approval: Ethical approval for this study was obtained from the Gazi University Ethics Committee (approval number and date 2021-445/06.04.2021).

Informed Consent: Informed consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: A.A., Design: A.A., B.K., Data Collection or Processing: A.A., B.K., Analysis or Interpretation: A.A., B.K., Literature Search: A.A., Writing: A.A., B.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Determination of Factors Influencing Pharmacists While Recommending Immune-Enhancing Products *via* Analytic Hierarchy Process

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ABSTRACT

Objectives: Immune enhancers are attracting attention day by day. Besides, during the coronavirus disease-2019 (COVID-19) pandemic, there has been an increasing demand for immune enhancers. Pharmacists are seen as trustable providers of complementary and alternative medicines, dietary and herbal supplements, immune-enhancers, and so on. This study aims to prioritization criteria that affect community pharmacists' recommending behavior regarding immune enhancers.

Materials and Methods: This paper adopts the analytic hierarchy process (AHP) to rank different criteria substantial for affecting community pharmacists' recommending behavior regarding immune enhancers. In this direction, firstly seven criteria were identified through literature review and views of pharmacists who have community pharmacy experiences. These are; (i) ease of access, (ii) selling price, (iii) package, (iv) content (appropriateness to patient health status), (v) expectation of patient, (vi) quality, and (vii) trust in the manufacturer. Then, a questionnaire including criteria was prepared and delivered to community pharmacists. The data obtained from 93 participants were transferred to the Super Decisions software. The hierarchical structure of the AHP was established and pair-wise comparisons were made.

Results: This study showed that the most important criterion was the ease of access (28%). Secondly, pharmacists give importance to the content of the product, while advising immune-enhancers (22%). Besides, it was determined that the least important criterion was the package of the product (4%).

Conclusion: This study will contribute to the literature by facilitating the process of assessing factors that pharmacists pay attention to while recommending immune-enhancing products. Additionally, the present study results will shed light on firms producing such products, to shape their supply chain management strategies, especially for marketing and sales.

Key words: Immune-enhancers, pharmacist, analytic hierarchy process

INTRODUCTION

Immunity plays a crucial role in protecting against harmful agents, particularly pathogenic organisms like bacteria, viruses, fungi, and parasites.[1] Recently, strong immunity has been thought of as an indicator of a healthy life. This situation also increased individuals' demand for immune-enhancing products. Additionally, it is seen that healthcare professionals recommend immune enhancers for diseases for which there is no definitive treatment yet.[2] The coronavirus disease-2019 (COVID-19) pandemic is one of the best examples of this situation. These products have also been commonly recommended to prevent COVID-19.[3]

Immune-stimulants are synthetic or biological-originated biomolecules that help regulate, suppress, and stimulate the immune system, including different product groups such as vitamins, minerals, probiotics, antioxidants, herbal, and dietary supplements (DS), and some of the complementary and alternative medicines.[4-6] Usage of these products is gradually

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increasing.[7,8] The safest place where these products are offered to the market is pharmacies. Besides, individuals trust pharmacists, while choosing them.[9-11]

In the literature, studies on these products mainly focus on pharmacists' roles, knowledge, and attitude.[12,13] Boon et al.[14] summarize pharmacists' role in natural health products/DS in three main topics as demand, safety issues, and accessibility from pharmacies.

The number of studies dealing with pharmacists behavior to recommend different immune-enhancing products is quite limited. In a qualitative study conducted in Australia, Culverhouse and Wohlmuth express that providing health benefit is the primary reason for recommending complementary medicines (CM) for pharmacists and state customer demand, company profile, and cost as some other factors.[10] According to Kanjanarach et al.[15], Thai pharmacists' selecting criteria of DS and CM are determined as the credibility of the firm, customer income, product appearance, and firm's approach to unsold products. A study was conducted with community pharmacists ranking the factors that influence the purchasing decision of medicines, and these factors are stated as customers' satisfaction, profitability, promotion, and original-generic drug price difference, respectively.[16]

In this regard, this study evaluates and prioritize factors, determined considering the above mentioned studies, which affect the community pharmacists' recommending behavior related to immune-enhancers in Türkiye. To the best of the authors' knowledge, this is the first study that prioritizes the pharmacists' selection criteria of immune-enhancers *via* the Analytic Hierarchy Process (AHP) approach.

MATERIALS AND METHODS

Within the scope of this study, prioritization of the factors affecting pharmacists' choice of immune enhancers for patients will be done with the AHP method. A questionnaire form was prepared according to the AHP approach in line with this purpose.

The AHP is one of the multi-criteria decision-making techniques based on pair-wise comparisons, developed by Saaty[17] in the 1970s. The AHP can be used in health management and patient-related issues.[18-20]

In this study, the hierarchical structure of the AHP was established and solved *via* the Super Decisions Support software. Criteria are identified according to the literature[10,14,15,21-26] and views of pharmacists, who have community pharmacy experiences. These are; (i) ease of access, (ii) selling price, (iii) package, (iv) content (appropriateness to patient health status), (v) expectation of patient, (vi) quality, and (vii) trust in the manufacturer (Figure 1).

The ethics committee permission was obtained from İzmir Katip Çelebi University Social Research Ethics Committee (04.09.2020-no: 2020/09-08). Online questionnaires were delivered to community pharmacists in Türkiye between September 10th, 2020 and October 19th, 2020. Participants were informed about the study and their consent was obtained.

The literature states that the sample size can be one or more, around 109 on average in AHP studies.[19] One hundred two community pharmacists answered the questionnaire in this study, despite working conditions during the COVID-19 pandemic. Nine of them were not included in the analysis due to a lack of answers. Therefore, 93 participants were considered.

RESULTS

The study takes arithmetical averages of pair-wise comparison matrices created by pharmacists. Table 1 summarizes the findings obtained from the pair-wise comparison matrices for the criteria.

As given in Table 1, calculated averages are rounded to the nearest integer. The information in Table 1 can be summarized as follows: "Ease of access" is about 3.769 (almost 4) times more important than "package", "selling price" is about 4.432 (almost 4) times more important than "package", "content" is about 6.403 (almost 6) times more important than "package", and "quality" is about 4.387 (almost 4) times more important than "expectations of patient" *etc*.

After that, data are transferred to the Super Decisions software. The priority values of the criteria and the consistency rate are calculated. The inconsistency rate is calculated as 0.093, which

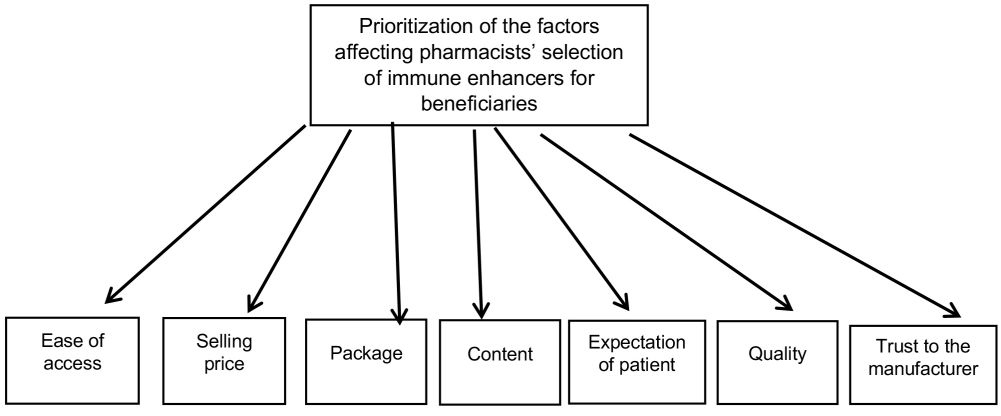

Figure 1. Hierarchical representation of the proposed issue

should be less than 0.1.[17] In this regard, this value is under an acceptable level of inconsistency. The priority values of the criteria are given in Table 2.

In Table 2, the "ease of access" is found as the most important criterion (28%). Three other criteria follow it: "content" (22%), "selling price" (19%), and "quality" (14%).

Table 1. Pair-wise comparisons of criteria

Criteria	Averages	Criteria
Ease of access	2.694	Selling price
Ease of access	2.276	Content
Ease of access	3.769	Package
Ease of access	2.209	Quality
Ease of access	2.109	Expectations of patient
Ease of access	2.450	Trust to the manufacturer
Selling price	2.121	Content
Selling price	4.432	Package
Selling price	2.099	Quality
Selling price	2.430	Expectations of patient
Selling price	2.369	Trust to the manufacturer
Content	6.403	Package
Content	2.731	Quality
Content	4.173	Expectations of patient
Content	3.785	Trust to the manufacturer
Quality	2.159	Package
Expectations of patient	2.222	Package
Trust to the manufacturer	2.230	Package
Quality	4.387	Expectations of patient
Quality	3.572	Trust to the manufacturer
Expectations of patient	2.900	Trust to the manufacturer

Table 2. Priorities of criteria

Criteria	Priorities
Ease of access	0.2752
Content	0.2167
Selling price	0.1859
Quality	0.1418
Expectation of patient	0.0785
Trust to the manufacturer	0.0589
Package	0.0431

DISCUSSION

The immune-enhancing products are generally included in over-the-counter (OTC) drugs. Pharmacist opinion highly influences customers' decisions, while selecting these products.[21] Moreover, Chan and Tran mentioned that individuals had viewed community pharmacies as preferable places for OTC products due to having the opportunity to access trustable information and safe products.[27] Therefore, it can be considered that pharmacists' influence in selling these products is essential. To the best of the authors' knowledge, this is the first study assessing community pharmacists' recommending behavior for immune-enhancers and revealing the order of importance of the criteria *via* the AHP. According to the study results, the most and least important criteria were the ease of access and the package.

Procurement of health products is a pharmacists' main functions according to Good Pharmacy Practice (GPP) Guidelines.[28] Additionally, today pharmacy practices change from product-oriented to patient-oriented. However, as stated by Moltó-Puigmartí et al.[22], it should be noted that patient-oriented service delivery's complete success depends on patients' access to the relevant product. In other words, pharmaceutical care services offered in pharmacies should be both product- and patient-oriented. In this context, accessibility to a product is of paramount importance. Especially, accessing OTCs such as immune-enhancers became more crucial during the COVID-19 pandemic. Indeed, considering the questionnaire applied in the current study during the COVID-19 pandemic, it should not be surprising that the first criterion becomes ease of access from the viewpoint of pharmacists.

Community pharmacists are closely concerned with their patients' health status *via* pharmaceutical care services. These services also balance selling a product and meeting patient healthcare needs. According to the clinical decision-making process, "identifying alternatives" and "choosing among alternatives" are included as main steps for pharmacists.[29] These steps affect pharmacists' recommending behavior of a product. Taking the medication history of a patient is a necessary process. An inaccurate or incomplete medication history can lead to negative consequences.[30] It is known that concomitant usage of drugs or herbal supplements with other medications can cause unwanted drug interactions.[31] This situation is vital, primarily when the pharmacist evaluates the drugs or non-pharmaceutical products used by the patients and offers the patient suggestions about these products. This is why immune enhancers content becomes an important factor after accessing these products. Pharmacists attach importance to selecting the most appropriate product for consumers/patients. Similarly, De Tran et al.[23] stated that, while recommending an OTC product, Vietnamese community pharmacists are most significantly influenced by the combination of active ingredients, range of dosage forms, and quick onset of action, related to the product. When selecting dietary/nutritional supplements, Nickerson-Troy et al.[24] expressed that pharmacists should pay attention to patient characteristics such as disease state and concomitant usage with medicines/supplements. Depending

on the most commonly cited bioethics principles, pharmacists should first consider the patient's benefit (beneficence) and prevent patients from being harmed (non-maleficence).[32,33] As Hanna and Hughes's[25] study emphasized, pharmacists should provide patient safety while offering an OTC to a patient. This is another indicator that evaluating product content regarding patient health status is critical, while pharmacists recommend immune-enhancers.

New resources are required for pharmacies' financial survival due to the regulations restricting health expenditures. In the literature, supporting non-prescription sales is seen as crucial for improving pharmacy economies.[21,34,35] The market size of natural health products/DS is increasing, and these products have good profitability for pharmacies.[14] In this study, considering the effect of the product-selling price on pharmacy profitability, sale price is one of the most important factors affecting pharmacists' recommending behavior related to immune-enhancers. The Turkish Pharmacists Association published a report addressing community pharmacies' economic and financial situation in Türkiye in 2019. According to this report, there is insufficient improvement in community pharmacies' economies.[36] This is an expected result, when considering community pharmacies' financial situations in Türkiye. In contrast, De Tran et al.[23] revealed that Vietnamese community pharmacists are least influenced by economic factors including financial pressure of excess stock, profit from the product, and volume selling product, while suggesting an OTC.[23]

According to Kanjanarach et al.[15], motivation to recommend DS/CM often comes from customer demands. Welna et al.[26] presented that the first two factors that affect community pharmacists' decisions about stocking natural products were patients' requests and the demand of consumers/popularity, respectively. Contrarily, in this study, patient expectation was not one of the primary factors for pharmacists.

Kanjanarach et al.[15] stated that Australian and Thai pharmacists consider firms' credibility, while selecting DS/CM. De Tran et al.[23] expressed that brand factor, including confidence in the manufacturer, was of medium importance among five factors about Vietnamese community pharmacists' OTC recommendation. Welna et al.[26] put forth that manufacturers' reputation and "willingness/ability to provide product quality data" were the first five of the eighteen criteria affecting pharmacies' natural product stocks. However, in this study, quality and trust in the manufacturer did not occur in the upper ranks. Considering the immune-enhancers sold in community pharmacies are strictly controlled and licensed, pharmacists' priorities may be affected by this. The package was the least important factor affecting pharmacists' behavior, while recommending immune-enhancers. It can be said that pharmacists do not pay much attention to the packages of immune-enhancer products. Similarly, in the study of Kevrekidis et al.[21], packaging was the least affecting factor for customers while selecting OTCs.

CONCLUSION

Product selection and recommending decisions of pharmacists can directly affect the health outcomes of patients. In this regard, using scientific methods to evaluate the decision process is vital. As it is known, pharmacists should be good decision-makers. In this study, one of the most widely used multi-criteria decision analysis techniques, AHP, was applied to investigate pharmacists' recommending behavior related to immune-enhancers. Examining the importance order of the criteria that affect pharmacists' recommending behavior related to immune-enhancers will fill the gap in the literature and contribute to the continuation of the services offered in the pharmacy without interruption.

The results obtained from this study raise several important issues that could spark further research, especially on pharmaceutical production, marketing, logistics, and public relations. A similar study design can be adapted for companies with no immune-enhancing products but other natural health products/supplements.

The study results may shed light on pharmaceutical educators developing curricula differently. The increased demand for supplements and OTCs, especially with COVID-19 pandemic, has revealed that pharmacists should know OTC and prescription drugs and manage these products correctly. For this reason, it is necessary to include these products in pharmacy faculty curricula. It is thought that the results of the study will be instructive about the points to be considered in presenting these products to patients.

Ethics

Ethics Committee Approval: The ethics committee permission was obtained from İzmir Katip Çelebi University Social Research Ethics Committee (04.09.2020-no: 2020/09-08).

Informed Consent: Participants were informed about the study, and their consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: N.T., Design: N.T., M.A., Data Collection or Processing: N.T., M.A., Analysis or Interpretation: M.A., Literature Search: N.T., M.A., Writing: N.T., M.A.

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REVIEW

Emerging Role of Biopharmaceutical Classification and Biopharmaceutical Drug Disposition System in Dosage form Development: A Systematic Review

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ABSTRACT

Biopharmaceutical classification system (BCS) is an advanced tool used for classifying medicines based on dissolution, water solubility, and intestinal permeability, which affect the absorption of active pharmaceutical ingredients (API) from immediate-release solid oral forms. It is useful to the formulation researchers to develop novel dosage forms based on modernistic rather than experimental approaches. The current review focuses on the fundamentals, objectives, guidance of BCS, characteristics of BCS drugs, their importance and applications of BCS. This review explains the challenges in drug development in terms of solubility and *in vivo* disposition. In the current review, new strategies for improving BCS II drug solubility as well as biopharmaceutical drug disposition properties which are utilized throughout the early stages of drug development and commercialization are mainly discussed.

Key words: Bioavailability, biopharmaceutical classification system, drug solubility, dissolution, drug disposition, bioequivalence, new drug application

INTRODUCTION

Biopharmaceutical classification system (BCS) is an advanced tool used for classifying drug substances on dissolution, intestinal permeability and water solubility.[1] In 1995, a theoretical approach for comparing *in vitro* drug dissolution with *in vivo* bioavailability was first conducted by Amidon et al.[1] BCS is a pharmaceutical development tool that is used for basic management in the drug discovery and early development of novel medications.[1,2]

The criteria for BCS direction for biowaiver are given by the United States Food and Drug Administration (FDA or USFDA), World Health Organization (WHO) and European Medicines Agency.[3,4] The BCS data assist the particular researcher in constructing a dosage form based on intuition rather than experimental approaches (FDA rules, 2000).[5] The BCS conceptual structure requirements can be linked to New Drug Application and Abbreviated New Drug Application approvals as well as scale up and post-approval alterations in medication manufacturing.

BCS is a conceptual structure that discusses three rate-limiting phases in oral retention.

- Release of drugs from dosage form
- Gastrointestinal (GI) tract arrangement of disintegrated form
- Saturation through GI membrane into hepatic circulation[6]

The intestinal permeability arrangement is determined by a correlation with intravenous infusion and solubility characterization is determined according to United States Pharmacopeia (USP).[7]

BCS follows fick's first law utilized for membrane permeability

$$J_f = P_m C_i \quad \text{(equation 1)}$$

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Where,

J_f = Drug flux rate (mass/area/time)

P_m = Membrane permeability

C_i = Concentration of the drug at the intestinal membrane surface

BCS acts as a regulatory tool and replaces certain bioequivalent studies, which are have been accurate *in vitro* dissolution tests and ensure avoiding unnecessary drug exposure to healthy volunteers.[8]

BCS classification

According to BCS system, drugs are classified into four types based on their intestinal permeability and solubility. BCS classification is based on key parameters like solubility, dissolution rate and permeability, which control absorption. In case of class I drugs, absorption is maximum, class II drugs are showing solubility limited, class III drugs have permeability limited, class IV drugs have poorly absorbed mentioned in Table 1.[6-8] Apparent permeability index (Papp) is the index used to assess the degree of permeability of drug substances. The permeability coefficient, which is a measure of flow to the drug concentration in the donor compartment. Papp of any drug substance can be calculated using *in vitro*, *ex vivo*, *in situ*, and *in vivo* techniques.[9]

Solubility

The amount of a substance that can be dissolved in a given amount of solvent is called solubility. A medicine that can be dissolved in 250 mL or less of water throughout a pH range of 1-8 is deemed an excellent dissolved pharmaceutical.[10]

Permeability

Permeability is the quality or state of being permeable. When a medicine has an absorption rate of more than 90% of the prescribed dosage and is stable in the stomach, it is termed an exception penetrable pharmaceutical.[11]

Dissolution rate

The process by which a solute dissolves into a solvent and produces a solution is known as dissolution. When 85% of the labeled quantity of drug substance dissolved in 30 min using USP equipment 1 at 100 rpm or apparatus 2 at 50 rpm in a volume of 900 mL buffer solutions (0.1 N HCl/pH 4.5 buffer/pH 6.8 buffer without enzymes), the drug product is regarded to have fast dissolution.[12-14]

Dimensionless parameter

BCS characterization is connected to medication dissolution and absorption display, which are essential factors for regulating medication absorption as a set of dimensionless numbers.[15,16] Drug properties and their corresponding dimensionless parameters and their significance related to them are highlighted in Table 2.

Absorption number (A_n) =
Average residence time/average absorption time (equation 2)

The dissolution number (DS_n) =
Average residence time/average dissolution time (equation 3)

Dose number (D_n) = Mass of drug/
uptake volume of 250 mL × drug solubility (equation 4)

Objectives

- The goal of BCS is to evaluate *in vivo* performance of medicinal products based on *in vitro* permeability and solubility data.[17]
- To provide techniques for categorizing medicinal products based on solubility and permeability properties as well as dosage form dissolution.
- We improved the efficiency of drug development and review processes by proposing a mechanism to perform clinical bioequivalence tests expandable.

Table 1. BCS classification system[6-8]

Class	Solubility	Permeability	*Papp (cm/sec)	*Q	Significance
I	High	High	P_{app} >10^{-5}	q ≤0.5	Well absorbed
II	Low	High	P_{app} >10^{-5}	q >1	Solubility limited
III	High	Low	P_{app} <2 C 10^{-6}	q ≤0.5	Permeability limited
IV	Low	Low	P_{app} <2 C 10^{-6}	q >1	Poorly absorbed

*P_{app}: Apparent permeability, *Q: Dose/solubility, BCS: Biopharmaceutical classification system

Table 2. Drug properties influencing absorption[6-8]

Drug property	Corresponding dimensionless parameter	Significance
Solubility	Dose number	Ideally, the dose ratio should be less than 1. Higher doses will increase the ratio and absorption less likely
Dissolution rate	Dissolution number	Ideally, dissolution number should exceed 1. In the case of solid dosage forms, a combination of inadequate solubility or excessive particle size or density can increase the time needed for full dissolution and reduce this ratio
Permeability rate	Absorption number	Ideally, absorption number should exceed 1. Longer absorption times resulting from lower permeability will reduce this ratio

Importance

To replace certain bioequivalent studies, BCS acts as a regulatory tool. It is applicable in both preclinical and clinical examinations. BCS can reduce the time and money for the immediate release orally administered drugs, which meet particular criteria; the FDA will allow a waiver for costly and tedious bioequivalence studies. It acts as a guiding tool for selecting the formulation of new dosage forms, development of various oral drug delivery systems.[18]

Class II drugs

BCS class II drugs have high permeability and low solubility. These medications have a high absorption number, but a small disintegration number. *In vivo* drug dissolution is then a rate limiting advanced step for absorption, except in very high dose numbers. These drugs have varied bioavailability and require improved solubility or dissolution to increase bioavailability. These compounds are suitable to outline the sustained release and controlled release formulations. *In vitro-in vivo* correlation applies normally to class II drugs. Based on solubility and permeability, drugs are classified into four types (class I to class IV) examples mentioned in Table 3.[18]

Biowaiver

Biowaiver is most commonly used in the administrative drug approval procedure, when the drug application is confirmed based on the proof of proportionality other than *in vivo* comparison testing. This waiver applies to both the pre- and post-approval stages. BCS-based biowaiver is applicable for immediate-release solid oral formulations containing the API approved by WHO.[19]

Biowaiver extension potential

BCS class II medicines are effective and completely absorbed, when taken orally. Class II drugs are weak acids with pKa values of ≤4.5 and intrinsic solubility (dissolvability of the unionized form) of ≥0.01 mg/mL, which are ineffectively dissolvable. At pH values typical of the fasted state in the jejunum (about pH 6.5), these medications will have a solubility of >1 mg/mL, produce about rapid and steady dissolution of the medication. Class II drugs are inadequately dissolvable at gastric pH, in which pH is considerably less than pKa because the small intestinal transit time is more consistent and when fasting longer than the gastric residence time (3 hr), drugs these physical characteristics will have enough time to dissolve. Class II drugs meet the permeability measure, biowaiver for products that break down quickly at the pH levels regularly in the small digestive tract, it has been suggested that BCS class II drugs have a biowaiver enhancement potential.[19]

Applications

Dissolution or solubility is the rate limiting factor in BCS II and it has a substantial impact on absorption and bioavailability. Lyophilization, micronization, microemulsion, inclusion of surfactants, solid dispersion, and use of complexing agents such as cyclodextrins; these are the methods used to improve solubility.[19-22] Zer-Os tablet innovation, soft gel, triglas, and nano-sized formulations are enhancement techniques, for example; nanocrystals, nanosuspension, and nanoemulsions are useful methods for increasing the solubility and bioavailability of low water soluble drugs[23-25] mentioned in Table 4.

Table 3. Examples of some model drugs as *per* BCS[10-14]

Class I	Class II	Class III	Class IV
Abacavir	Amiodarone	Acyclovir	Amphotericin
Acetaminophen	Atorvastatin	Amiloride	Chlorthalidone
Acyclovir	Azithromycin	Amoxicillin	Chlorothiazide
Amiloride	Carbamazepine	Atenolol	Colistin
Amitryptyline	Carvedilol	Bisphosphonates	Coenzyme Q10
Antipyrine	Chlorpromazine	Bidisomide	Ciprofloxacin
Atropine	Cisapride	Captopril	Ellagic acid
Buspironec	Ciprofloxacin	Cefazolin	Furosemide
Caffeine	Cyclosporine	Cetirizine	Hydrochlorothiazide
Captopril	Danazole	Cimetidine	Mebendazole
Chloroquine	Dapsone	Cipofloxacin	Methotrexate
Chlorpheniramine	Diclofenac	Cloxacillin	Neomycin
Cyclophosphamide	Diflunisal	Dicloxacillin	Ritonavir
Desipramine	Digoxin	Erythromycin	Saquinavir
Diazepam	Erythromycin	Famotidine	Taxol

BCS: Biopharmaceutical classification system

Techniques to enhance the solubility of BCS II drugs

Physical modifications

Micronization: Spray drying or use fluid energy or a jet mill to reduce the particle size to 1-10 microns. A reduced particle size will increase the surface area and improves bioavailability. Examples: Griseofulvin, sulfa, and certain steroidal drugs.[26]

Nanoionization: Powdered drug is converted to nanocrystals of size 200-600 nm using technologies such as pearl processing, homogenization in water, and homogenization using non-aqueous medium. Examples: estradiol, doxorubicin, cyclosporin, and paclitaxel.[27]

Sonocrystallization: Ultrasound in the range of 20 KHz-5 KHz is used to induce crystallization in sonocrystallization. Examples: This method increased the solubility of ketoconazole by 5.517 folds.[28]

Use of polymorphs, amorphous, solvates, and metastable form: Because the vitality required to transfer the crystal lattice is more than that necessary for amorphous solid, amorphous forms are more soluble than crystal structures. Metastable forms are more soluble than stable ones. Because hydrates are already associated with water, anhydrates are more soluble, so require less energy for crystal separation. Thus, the order of solubility of different solid forms of drugs is

Amorphous › Metastable › Stable › Anhydrates › Hydrates › Solvates › Non-solvates

Eutectic mixtures: The soluble carrier in the eutectic mixtures dissolves when exposed to water, leaving the drug in a microcrystalline state that solubilize rapidly. They are inexpensive and easily prepared. Examples: paracetamol with urea, griseofulvin with urea, griseofulvin with succinic acid.[29]

Table 4. Techniques employed for BCS II drugs

Drug name (category)	Polymers/co-formers	Method employed	Result	References
Meloxicam (NSAIDS)	PVP, PEG-6000	Solvent evaporation method	Increase the dissolution rate	26
Etoricoxib (NSAIDS)	Lactose, sucrose, mannitol	Solvent evaporation method	Improved solubility and dissolution of the poorly aqueous soluble drug	27
Ibuprofen (NSAIDS)	Starch 1500, PVP K30	Kneading method	Developed faster dissolution characteristics	28
Diacerein Antirheumatic	PVP K30, HPMC-E4	Solvent evaporation	Improved solubility of poorly soluble drug	29
Itraconazole Antifungal	Gelucire 50-13, compritol 888 ATO	Spray drying	Increased dissolution and *in vivo* bioavailability	30
Griseofulvin Antifungal	Britishgum, corn starch	Roll mixing method	Solubility and dissolution rate increases	31
	Beta-cyclodextrin	Complexation using co-precipitation method	Enhanced dissolution rate was observed	32
Carbamazepine Anticonvulsant	Croscarmellose, sodium starch glycolate	Modified solvent evaporation method	Improved solubility/dissolution profile of drug	33
Glipizide Antidiabetic	HPMC, croscarmellose	Solvent evaporation	Better phase solubility and *in vitro* dissolution rate	34
Olanzapine Antipsychotic	Pregelatinized starch, sodium starch glycolate	Dispersion method	Enhanced the aqueous solubility	35
Gliclazide Oral hypoglycemic agent	PEG 4000, PEG 6000, PVP K-30	Fusion and solvent evaporation method	Increased solubility and bioavailability rate of poorly soluble drug	36
Atorvastatin Antihyperlipidimic agent	Mannitol, PEG 4000, PVP K-30	Hot melt and solvent evaporation	Improved dissolution rate	37
Telmisartan Antihypertensive	Beta-cyclodextrin, MCC pH 102, polaxomer 188	Solid dispersion method	Increased solubility, dissolution and bioavailability	38
Mesalamine (antiulcerative)	SLS, urea	Kneading method	Improved saturation solubility and dissolution rate	39

NSAID: Non-steroidal anti-inflammatory agent, PVP: Polyvinylpyrrolidone, PEG: Polyethylene glycol, HPMC: Hydroxypropyl methylcellulose, BCS: Biopharmaceutical classification system, SLS: Sodium lauryl sulfate

Solid dispersions: A hydrophilic matrix (polyvinylpyrrolidone, povidone, polyethylene glycol, surfactant such as sodium lauryl sulfate, tween 80, pluronic F-68) and hydrophobic drug (fats, oils, waxes, alkanes, and other greasy substances) are used in preparing solid dispersions. Methods for preparing solid dispersions including.

Hot-melt method (fusion method): Drug and the carrier are heated directly until they melt and then rapidly cooled with ice by continuous stirring to solidify. After that, it is crushed, pulverized, sieved and compressed into tablets.[30]

Solvent evaporation method: Medication and the carrier were dissolved in a common solvent and the dissolved content was evaporated under vacuum to form an amorphous precipitate.[30] Examples: Meloxicam, naproxen, nimesulide.[31]

Hot melt extrusion: It is the same as the combination technique, except the extruder does the extreme mix. It is appropriate for large-scale preparations.[31] Examples: Ritonavir.[32]

Chemical modifications

Change in pH: The easiest approach to enhance solubility of organic ionized solutions is to change the pH of the formulation. A change in pH can be done by;

- Use of buffers
- *In situ* salt formation

Salt formation: When compared with pure API drugs, salt forms have better solubility. Example: Antacid metal salts of acidic medicines, such as penicillin, solid corrosive salts of vital pharmaceuticals, such as atropine.[33]

Prodrug: Solubility of the drugs can be increased by converting a pharmacologically inactive substance into a pharmacologically active drug. Examples: acyclovir, fluorouracil, cyclophosphamide, carbamazepine, captopril, and carisoprodol.

Atomic elucidation with cyclodextrins: The beta and gamma ray cyclodextrins can form sub-atomic consideration structures since they have a cavity to accommodate lipophilic medicines as guests and the exterior of the transporter is hydrophilic. As a result, there is a significant increase in dissolving rate and solubility. Thiazide diuretics, barbiturates, and benzodiazepines are examples of drugs with enhanced bioavailability due to this method.[34]

Derivatization: Conversion of a chemical compound into a product, which shows a similar chemical structure called derivative with different solubilities's that of the adduct.[35]

Miscellaneous modifications

Super critical fluid (SCF) recrystallization: These fluids have temperatures and pressures that are higher than their critical temperature and exhibit the characteristics of both gases and liquids. SCFs are profoundly compressible at close fundamental temperatures, modifying thickness and mass power by allowing weight modification. When the drug particles were dissolved in SCF, they crystallized with smaller molecule sizes.[36]

Use of surfactants: Surfactants increase the disintegration rate by advancing wetting and infiltration of disintegration liquid into the medication particles, when used in the focus beneath their basic micelle fixation because drug captured in the micelle structure failed to partition in the dissolution fluid above the critical micelle concentration. Example: a steroid-like spironolactone bioavailability has been enhanced by this technique.[36-38]

Solvent deposition: Poorly soluble medicines are dissolved and deposited on an inert, hydrophilic, and solid matrix by evaporation of the solvent using organic solvents such as alcohol. Example: Nifedipine.

Precipitation: Medication that is poorly water-soluble is first dissolved in a suitable organic solvent, then quickly mixed with a non-dissolvable to precipitate the medication in nanosize particles, and this result is known as a hydrosol.[39] Example: cyclosporine.

Co-solvents: Solubility is low for weak electrolytes and non-polar compounds. Solubility can be increased by altering the polarity of those molecules by adding organic co-solvents (mixing miscible or partially miscible solvents) to water, which drastically affects medication solubility.[40] Example: Etoricoxib, glipizide, glyburide, glimepiride and pioglitazone.

Hydrotrophy: The addition of a significant number of additives (hydrotropic agent) to the drug solution increases the medication's water solubility.[41] Example: Ethanol, resorcinol, pyrogallol, catechol and procaine hydrochloride.

Selective adsorption on insoluble carriers: Adsorbents can enhance solubility by forming weak physical bonding between the drug and adsorbent and can also by hydration and swelling of clay in aqueous media. Example: inorganic clay bentonite can improve the dissolution of drugs like griseofulvin, prednisone, and indomethacin.[42]

Drug disposition

The significant route of elimination of drugs showing high intestinal permeability in humans is mainly by metabolism and the drugs having weak intestinal permeability rates are mainly excreted as unchanged drugs in the urine and bile in humans. In 2005 drug disposition was first observed by Wu and Benet[43], who proposed a system called Biopharmaceutics Drug Disposition Classification System (BDDCS): in case of class 1 and 2 drugs showing extensive metabolism, class 3 and 4 drugs showing a poor metabolism rate shown in Table 5.[43-45]

BDDCS system estimates the effect of food, absorption as well as efflux transporters, route of excretion on overall drug absorption and the permeability of immediate-release oral dose forms is less than bioavailability. BDDCS system as an extension of BCS.[44]

Because BDDCS is a replacement for permeability, they proposed that medications that demonstrate metabolism as a main route of elimination be deemed highly permeable. Low permeable drugs are those, whose primary route of excretion is renal and biliary excretion of unmodified medicine.[45] Data on medication disposition for a few medicines from the WHO essential drug list are shown in Table 6.[46]

Table 5. Biopharmaceutics drug disposition classification system[43-45]

	High solubility	Low solubility
Extensive metabolism →	**Class 1** High solubility Extensive metabolism	**Class 2** Low solubility Extensive metabolism
Poor metabolism →	**Class 3** High solubility Poor metabolism	**Class 4** Low solubility Poor metabolism

Table 6. Drug disposition data from WHO essential medicines list[46]

Model drug	Dose (mg)	Formulation	Solubility (mg/mL)	Dose number	Bioavailability (%)	Excreted unchanged in urine (%)	Metabolism	BCS	BDDS
Aspirin	500	Tablets	10	0.2	Limited data	1.4	Extensive	3	1
Benznidazole	100	Tablets	0.4	1.0	96	NA	Extensive	3	1
Biperiden	2	Tablets	1.0	0.008	36	NA	Extensive	3	1
Clomiphene citrate	50	Tablets	1.11	0.2	90	8.0	Poor	1	3
Didanosine	25	Tablets	27.3	0.004	44	55	Poor	3	3
Ethambutol	400	Tablets	100	0.016	Not applicable	79	Poor	3	3
Ethosuximide	250	-	100	0.01	Not applicable	NA	Extensive	3	1
Folic acid	1	-	0.1	0.04	Not applicable	NA	Poor	3	3
Glibenclamide	5	-	0.01	16	Not applicable	NA	Extensive	2	2
Levothyroxine sodium	0.1	-	0.15	0.003	70	NA	Poor	1	3
Lumefantrine	120	-	1	0.48	Not applicable	NA	Poor	1	3
Methyldopa	250	Tablets	10	0.1	25	40	Extensive	3	1
Nicotinamide	50	-	100	0.002	High	NA	Extensive	3	1

NA: Not available, WHO: World Health Organization, BCS: Biopharmaceutical classification system, BDDS: Biopharmaceutical drug disposition system

CONCLUSION

BCS serves as a regulatory tool for the progress of various oral drug transport advancements. The BCS considers three major factors, dissolution, solubility and intestinal permeability, which govern the rate and degree of medication absorption from immediate solid dosage forms. It is a controlling device for anticipating *in vivo* execution of the medicinal substance and the improvement of the medication delivery system. The data generated from the solubility and permeability in pipeline drug discovery or development can be used for early pipeline compound categorization. The BCS's advantageous circumstances include reduced medication exposure to a large panel of human participants and in some cases shorter drug product development time, in addition to significant cost savings.

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Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: R.S., Design: R.S., J.C., Data Collection or Processing: R.S., S.K., Analysis or Interpretation: R.S., J.C., Literature Search: R.S., S.K., Writing: R.S., J.C., S.K.

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REVIEW

Evidence for Health-Promoting Properties of *Lepidium sativum* L.: An Updated Comprehensive Review

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ABSTRACT

Lepidium sativum L. is a common herb distributed worldwide, used as a food ingredient and therapeutic agent in traditional medicine for treating health-related disorders. *L. sativum* and its extracts have been described to possess numerous biological activities including antimicrobial, antidiabetic, antioxidant, antidiarrheal, anticancer, and numerous health-promoting effects in *in vivo* and *in vitro* studies. The purpose of this review is to summarize the findings describing important biological functions and therapeutic effects of *L. sativum* in various cell lines and animal models. In this review, the English-language articles were gathered from electronic databases including Web of Science, PubMed and Google Scholar with no time limit applied to any database. The search terms used in this review include, "*Lepidium sativum* L." and/or "chemical composition", "health benefits", "antimicrobial", "antioxidant", "anticancer", "diuretic", "nephro-protection", "antidiarrheal", "antidiabetic", "anti-asthmatic", "neuroprotection", "metabolic", "bone fracture", and "reproductive performance". Additional and eligible studies were collected from reference lists of appropriate articles. The information presented will be helpful to attract more interest toward medicinal plants by defining and developing novel clinical applications and new drug formulations in the future. Pre-clinical studies showed that *L. sativum* possesses potent health-promoting effects involving various molecular mechanisms. Taken all together, data suggested that identified herbal plants such as *L. sativum*, can be exploited as nutritional and therapeutic agents to combat various ailments. Despite much research in this field, further comprehensive *in vitro*/*in vivo* studies and clinical trials are needed to identify the mechanisms underlying the biological and therapeutic activities of *L. sativum*.

Key words: Ethnomedicine, medicinal plants, *Lepidium sativum*, nutraceutical, therapeutic agents

INTRODUCTION

Many plants have been considered a principal source of potent therapeutic drugs for centuries. *Lepidium sativum* L., (alias Garden cress) is a fast-growing perennial herb with edible leaves that grows up to 50 cm in height and belongs to the family Brassicaceae (Cruciferae).[1-3] It is widely dispersed throughout the world; Africa, Asia, Australasia, Europe, Northern, and Southern America.[4-6] The leaves and seed oils are commonly applied in traditional medicine to treat various clinical complications, including asthma, hypertension, hyperglycemia, hepatitis, menstrual problems, sexual debility, arthritis, fracture, diarrhea, vitamin C deficiency, constipation, and migraine.[2,7,8] Additionally, they have been represented with pharmacological properties such as immunity booster, anticancer, antioxidant, laxative, febrifuge, diuretic, and galactogogue activities (Figure 1).[1,9-11]

The chemical composition of the *L. sativum* seeds illustrated that they contain high levels of proteins, fatty acids (oleic and linolenic acids), crude fiber (lignans, *etc.*), essential minerals (potassium, phosphorus, calcium, and iron), phytosterols (sitosterol, campesterol, and avenasterol), carotenoids, alkaloids (lepidine, *N*, *N'*-dibenzylthiourea, *N*, *N'*-dibenzyl urea,

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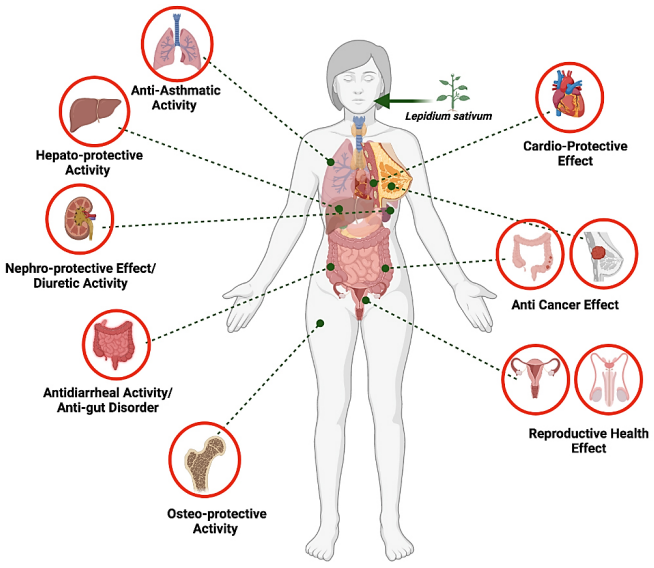

Figure 1. Therapeutic effects of *Lepidium sativum* under various physiological/pathological conditions

sinapine), hydroxycinnamic acids (sinapic acid),[12] glucosinolates (glucotropaeolin and 2-phenyl ethyl glucosinolates)[13] riboflavin, ascorbic acid, and tocopherols.[1,2,14-16]

Toxicological studies state that *L. sativum* seeds are considered practically non-toxic and safe.[17] Nowadays, ethnomedicinal studies gained great attention due to their beneficial roles against various ailments, though proper identification and documentation of medicinal plants seems indispensable.[18,19] Here, we provided an updated comprehensive overview of the chemical composition of *L. sativum* with focusing on its beneficial impacts, medicinal utility, and underlying mechanisms.

Chemical composition of L. sativum

Various factors (including variety, plant agronomic practices, seed collection stage, and geological conditions) contribute to the differences of the chemical composition of *L. sativum* seeds.[20] *L. sativum* seeds consist of carbohydrates, protein, lipids, and fiber.[21] *L. sativum* seeds also contain mucilaginous substances (cellulose and uronic acid containing polysaccharides). Additionally, the seeds have high protein and lipid contents, minerals as well as vitamins (*i.e.* vitamins A, C, D, B6, and cobalamin).[22] The presence of numerous components has been reported in *L. sativum* seeds including alkaloids, phenolic compounds, anthraquinones, and cardiac glycosides, flavonoids, tannins, benzoic, dihydroxybenzoic, gallic, chlorogenic, 4-hydroxycoumaric, vanillic, and salicylic acids, pyrogallol, catechin, caffeine, isoleucine as well as different imidazole alkaloids *e.g.* lepidine and semilepidine.[20] Essential (leucine, valine, lysine, phenyl alanine, isoleucine, arginine, histidine, threonine, and methionine) and non-essential (glutamic acid, aspartic acid, glycine, proline, serine, alanine, and tyrosine) amino acids are present in *L. sativum* seeds. Most abundant fatty acid in *L. sativum* seeds is α-linolenic acid (ALA), however, oleic, palmitic, stearic, arachidic, linoleic, lignoceric, behenic, acids β-sitosterol are found in different concentrations. *L. sativum* seed oils comprise high amounts of γ-tocopherol as well as α-tocopherol. Benzylcyanide and benzyl isothiocyanate are detected as significant volatile components of the seeds.[20,23] In a recent research, the results of the liquid chromatography-mass spectroscopy (LC-MS) of *L. sativum* extracts demonstrated various secondary metabolites (including kaempferol, apigenin, luteolin, quercetin, and 7-hydroxy-4',5,6-trimethoxyisoflavone, chlorogenic acid, sinapic acid, ascorbic acid, *p*-coumaric acid, 6-prenylnaringenin, and α-tocopherol) in different concentrations.[24]

Beneficial effects of L. sativum

Antimicrobial activity

The growth of antibiotic resistance in bacterial strains and adverse effects of synthetic antibiotics provide a route to exploiting plants with strong medical potential in treating bacterial infections.[2] Many studies have reported that the *L. sativum* extract is effective against bacterial strains and pathogens. It has been proposed that the antibacterial potency of *L. sativum* depends on benzyl isothiocyanate presence.[25] An increasing number of studies confirmed *L. sativum*'s antimicrobial properties (Table 1), however, exact mechanism of action, which elucidates how they could perform such activities has not fully understood. Recently, Al-Otaibi et al.[26] revealed probable therapeutic potential of the methanolic extract of *L. sativum* seeds in *Trypanosoma evansi* (a parasitic protozoan) infected-Swiss albino mice. Their findings showed that the methanolic extract treatment results in restoring the hematology analysis (haemoglobin content, hematocrit, erythrocyte count, leucocyte count, and percentage of lymphocytes) to the pre-infection values. Besides, the study discovered that the intraperitoneal (*ip*) injection of the extract exerts more efficacy rather than oral administration.[26] In another research it has been shown that the leaf extract of *L. sativum* could inhibit the viability of the protoscolices *Echinococcus granulosus*.[27]

Al-Marzoqi et al.[28] examined the antimicrobial potential of the crude alkaloid, phenolic, and terpenoid compounds of *L. sativum* extract. Their findings demonstrated that both Gram-positive and Gram-negative pathogens (*Staphylococcus aureus, S. epidermedis, S. saprophyticus, Klebsiella, Serratia, Proteus, Escherichia coli, Pseudomonas*, and *Provedenatia*) were resistant to phenolic compounds, whereas the alkaloid and terpenoid compounds exerted an extensive antimicrobial activity against Gram-positive and Gram-negative bacteria. Over all, they suggested that the hydrophobicity of components of the plant extracts, leads to disruption of bacterial cell membrane lipids and mitochondria that in turn causes microbial death.[28] They also showed that different concentrations of active components of the aforementioned plant demonstrated diverse effects on various pathogenic organisms.[25]

Table 1. Antimicrobial activity of various extracts of *Lepidium sativum*

Lepidium sativum	Antimicrobial activity	References
Seed petroleum ether, methanol, and water extracts	*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonae*, *Proteus vulgaris*, *Pseudomonas aeruginosa,* and *Candida albicans*	2
Seed extract	*K. pneumonia*, *E. coli*, *S. aureus,* and *Bacillus cereus*	7
Seed extract	*S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. enterica*, *K. pneumoniae*, and *C. albicans*	29
Chloroform, methanol extract	*E. coli*, *S. typhi*, *S. aureus*, *B. subtillis, Aspargillus niger, Fusarium oxysporum,* and *Fusarium solani*	30
Ethanol, methanol and chloroform extracts	*E. coli, K. pneumoniae, P. aeruginosa, S. aureus,* and *Shigella sonnie*	31
Seed methanol, and ethyl acetate extracts	*Rhodococcus equi*	32
Whole plant water, methanol and ethanol extracts	Multi-drug resistant *E. coli*	33
Crude extract of seeds	*P. aeruginosa, E. coli, S. aureus, B. subtilis,* and two fungi species *(Aspergillus niger* and *C. albicans)*	34

Antioxidant activity

Formation of reactive oxygen species (ROS) is provoked through normal metabolism, however, excessive amounts are detrimental and should be scavenged to avoid any damage.[35] Oxidative stress is implicated in the pathogenesis of several chronic ailments such as cancer, cardiovascular disease, and *etc*.[36] Intake of nutraceuticals (rich in antioxidants) from different herbs possibly protect human body against free radicals, thus, alleviate oxidative damage and degenerative diseases.[36] In a study conducted by Aydemir and Becerik[36], *L. sativum* seed extract exhibited antioxidant activity. In another study, antioxidant activity of *L. sativum* seed oil (petroleum ether) was evaluated employing free radical (2,2-diphenyl-1-picryl-hydrazyl, DPPH) scavenging activity method. The petroleum ether extract exerted antioxidant activity dose-dependently.[29] Omer et al.[34] reported that the ethyl acetate fractions of the *L. sativium* seeds had highest antioxidant activity. Another study conducted by Malar et al.[37] revealed the substantial antioxidant activity of the ethanolic extract of *L. sativum* plant parts (shoot, leaf, stem, and seed). Further, it has been shown that the presence of flavonoid and tannin in the methanolic extract of *L. sativum* leads to the significant antioxidant activity.[32]

The cyanobacterial toxins and crude extract provoke oxidative stress response in *L. sativum* seedlings through lipid peroxidation, elevation of the levels of tocopherol and antioxidant enzymes (including glutathione peroxidase, glutathione *S*-transferase, and glutathione reductase).[35] Furthermore, in a study performed by Kasabe et al.[38] it has been elucidated that the seeds of *L. sativum* possess antioxidant activities due to total phenolic content of the seeds. In a recent study, it was reported that methanolic extract of *L. sativum* exerted potent radical scavenging activity comparing to the ethanolic extract.[23]

Anticancer activity

Cancer has remained as the leading cause of death worldwide. Nowadays, applying natural remedies to overcome the side effects of conventional methods in cancer treatment have received growing attention.[39,40] *L. sativum* demonstrated anticancer, antiproliferative and cytotoxic effects through different mechanisms such as induction of apoptosis and necrosis in various cancer cells. An *in vitro* study carried out on breast cancer cell line (MCF-7), reported the apoptosis induction capability of the aqueous extract of *L. sativum* seeds. However, high concentrations of the extract resulted in necrosis.[41] Recently, El Sayed et al.[42] reported the antioxidant and anti-mutagenic effects of *L. sativum* against *in vivo* Ehrlich ascites carcinoma (EAC) in Swiss albino mice. Their findings demonstrated anticancer effect of *L. sativum* in EAC tumor-bearing mice lifespan. Additionally, increased levels of liver enzymes and glutathione peroxidase activity as well as decreased levels of malondialdehyde (MDA) were observed, which in turn indicated the antioxidant properties of the extract. Besides, *L. sativum* extract decreased chromosomal aberration and DNA fragmentation.[42] In another study conducted by Selek et al.[43], *L. sativum* methanolic extract substantially induced apoptosis in human peripheral lymphocyte cells, colon cancer (DLD-1), and endometrium cancer cell lines (ECC-1) in a dose-dependent pattern, besides, the extract presented significant antioxidant activity. Taken all together, they suggested that high levels of phenolic and flavonoid compounds of the extract may be considered as the underlying mechanism for the anticancer activity of *L. sativum*.[43] The antiproliferative effects of the leaf aqueous extracts of *L. sativum* were explored on human tongue squamous carcinoma (CAL-27). The aqueous extract inhibited growth of CAL-27 cells concentration-dependently. Apoptosis induction and DNA damage was observed in *L. sativum* extract-treated cancer cells. ROS generation in the mitochondria of the treated cells seems the cause of apoptosis induction.[44] According to a recent study, the hydroalcoholic extract of *L. sativum* showed cytotoxicity on HeLa cell line.[45] Further, Aslani et al.[46] evaluated cytotoxic effects of hydro-alcoholic extracts of *L. sativum* shoots in K562 cell line as a model of CML. MTT assay results depicted that the extract exerted cytotoxic effect on K562 cell line in a dose and time dependently.[46]

Effects on urinary system

Diuretic activity

There are different studies suggested that the plant's diuretic effect may depend on presence of phytochemicals such as flavonoids, saponins, steroids or organic acids.[47] In line with this, Patel et al.[47] illustrated that the aqueous and methanolic extracts of *L. sativum* dose-dependently augmented urine secretion in rat models. They suggested that *L. sativum* extracts' diuretic activity possibly induced by individual or synergistic effects of flavonoids and steroids, which in turn leads to increased local blood flow and vasodilation or inhibition of water and anions tubular reabsorption.[47] In addition to excessive urine production, increased sodium and water excretion contributes to *L. sativum*'s antihypertensive effect.[47] Maghrani et al.[48] investigated diuretic and antihypertensive properties of the aqueous extract of *L. sativum* in normotensive and spontaneously hypertensive rats (SHR). Oral administration of the extract caused substantial drop in blood pressure as well as increase of urinary exertion of sodium, potassium, and chlorides in SHR rats.[48]

Nephroprotective effect

Numerous evidence elucidates some medications have potential to induce nephrotoxicity and acute renal failure which causes loss of renal functions. Phytochemicals of *L. sativum* may have antioxidant activity, which, thus, overcome the drug-induced nephrotoxicity.[49] In this regard, Yadav et al.[49] depicted that the ethanolic extract of *L. sativum* exerts nephroprotective and curative activity against cisplatin-induced nephrotoxicity in Wistar rats. The administration of the extract markedly declined the levels of urea, creatinine as well as lipid peroxidation and enhanced glutathione (GSH) levels.[49] Recently, it has been presented that *L. sativum* seed aquatic extract could ameliorate oxidative stress induced by dexamethasone in rats. Dexamethasone administration led to the elevation of thiobarbituric acid reactive substances (TBARS), hydrogen peroxide, liver function biomarker levels, and lactate dehydrogenase (LDH) activity. However, enzymatic and non-enzymatic antioxidants, protein content, and alkaline phosphatase (ALP) activity were markedly reduced. The aquatic extract administration in rats received dexamethasone, could alleviate lipid peroxidation, antioxidant status, and biochemical indices, when compared to the dexamethasone-treated group.[50] Furthermore, administration of *L. sativum* powder to the gentamicin-induced nephropathy in diabetic albino rats caused a substantial reduction of the serum levels of glucose, MDA, augmentation of the glutathione transferase (GST), superoxide dismutase (SOD), total antioxidant capacity (TAC), glutathione pyroxidase (GPX), and catalase (CAT) activity as well as serum insulin levels, though exerted nephoprotective effect by enhancing renal damage.[51]

Effects on digestive tract

Antidiarrheal activity

A few studies reported the antidiarrheal and antispasmodic properties of *L. sativum*.[9,52] In a study performed in rat model, the administration of extract can inhibit castor oil-induced diarrhea like dicyclomine. Data from the study proposed that dual suppression of muscarinic receptors and Ca^{2+} channels was responsible for the antidiarrheal/antispasmodic activities of the *L. sativum*. Moreover, presence of gut relaxant compounds and phytochemicals such as alkaloids, and β-sitosterol, plays an important role in *L. sativum* antidiarrheal/antispasmodic effect.[9] Additionally, they examined antidiarrheal/antispasmodic properties of the crude extract of *L. sativum* seeds in multiple species (mice, Sprague-Dawley rats, guinea-pigs, and local breed rabbits). They also depicted the antidiarrheal/antispasmodic mechanisms specific to each species as below: 1) in rabbit model: activation of K^+ channels and blockade of PDE enzyme, 2) in guinea-pig model: anti-muscarinic and weak Ca^{2+} antagonist-like pathways and 3) in rat model: a combination of anti-muscarinic, Ca^{2+} antagonist and PDE-inhibitory-like mechanisms.[53]

Effect on gut disorders

The aqueous-methanolic extract of *L. sativum* seeds were reported to be potent contributors to indigestion and constipation (as digestive disorders). Najeeb-Ur-Rehman et al.[54] described an *in vivo* experiment conducted in mouse model displaying the atropine-sensitive pro-kinetic and laxative properties, which were relatively mediated through muscarinic receptors.

Metabolic activity

Findings of a recent study depicted that ethanolic and aqueous extracts of *L. sativum* significantly exerted hepato-protective, hypolipidemic, hypoglycemic, hypoinsulinemic, anti-obesity, antioxidant, and anti-inflammatory properties in high fat diet-fed rats. Moreover, the hepatic tissues of ethanolic/aqueous extracts-treated rats demonstrated upregulation of the intracellular phosphorylation of common markers of insulin signaling cascade (*p*-IR/p-AKT/pmTOR/p-p70S6K). Both extracts mitigated lipid peroxidation and restored the amounts of antioxidant enzymes.[55] Al-Asmari et al.[56] designed a study to assess the hepatoprotective effect of ethanolic extract of *L. sativum* against carbon tetrachloride (CCl_4)-induced toxicity in rat model. Their findings demonstrated that the level of serum alanine transaminase (ALT), ALP, aspartate transaminase (AST), and bilirubin was significantly decreased in ethanolic extract-treated rats. Additionally, histological analysis of liver tissues exhibited mild necrosis and inflammation in extract-treated group in comparison to the CCl_4-treated group.[56]

Similar findings were obtained by Zamzami et al.[57] for hepatoprotective effects of *L. sativum* seeds against CCl_4-induced hepatic injury in New Zealand rabbits. The extract-treated rabbits showed significant reduction in serum levels of liver biomarkers (transaminases, γ-GT, and ALP), total bilirubin, cholesterol, triglycerides (TG) and elevated levels of total protein and albumin. Moreover, *L. sativum* extract reduced oxidative stress in liver tissues. Overall, biochemical analysis as well as histopathological examination revealed that *L. sativum* extract effectively could reverse the hepatotoxicity of CCl_4 *in vivo*.[57] In another study, potential protective and therapeutic effects of *L. sativum* against aluminum-induced injury of liver and kidney in albino rat were investigated. Data

from this experiment exhibited that administration of the extract led to a marked reduction in levels of serum biomarkers of liver (*e.g.* AST, ALT, ALP, bilirubin, urea, and creatinine) and kidney functions. It is also significantly augmented total protein and albumin. Besides, rats fed with the extract reversed necrosis of hepatocytes, glomeruli, and renal tubules. It has been suggested that the antioxidant properties of *L. sativum* seeds exerted the aforementioned beneficial effects.[58]

L. sativum seed powder exhibited the potent cardioprotective effect against 5-fluorourasil (FU)-induced cardiotoxicity and oxidative stress in albino rats. *L. sativum* seed powder significantly reduced the inflammatory markers [myocardial IL-1β and myeloperoxidase (MPO) activity], concentration of serum cardiac biomarkers (CK-MB and cTnI), whereas it increased gluthathion (GSH) concentration. Moreover, in the *L. sativum*-treated group, the hypertriglyceridemia and hypercholesterolemia factors were returned to the normal status compared to the 5-FU-induced cardiotoxicity group.[59] In another research, hypolipidemic activity of *L. sativum* seed extract against triton x-100 and high cholesterol diet (HCD)-induced hyperlipidemia was investigated on rats. Their results showed that the extracts significantly protected against all parameters [total cholesterol (TC), TG, low density lipoprotein cholesterol (LDLc), very low density lipoprotein cholesterol (VLDLc)] of HCD diet-induced hyperlipidemia, thus, may exert anti-hyperlipidemic effect.[60] Additionally, Raish et al.[61] evaluated hepatoprotective effect of *L. sativum* ethanolic extract in rat model with liver damage induced by D-galactosamine/lipopolysaccharide. Data from their study revealed that the extract significantly down-regulated the pro-inflammatory cytokines (*e.g.* TNFα and IL-6 mRNA), stress genes (iNOS and HO-1) and up-regulated the IL-10 expression dose-dependently. Furthermore, the extract pretreatment leads to down-regulation of nuclear NF-κB (p65), NF-κB-DNA binding activity, MPO activity, and nitric oxide (NO) level. Additionally, it can down-regulate caspase 3 and up-regulate Bcl-2 protein expression, which overall indicated that *L. sativum* markedly alleviates hepatic damage through reduction of oxidative stress, inflammation, and apoptosis in the liver.[61] Administration of *L. sativum* seed ethanolic extract effectively could ameliorate triacylglycerol, LDLc, and TC as well as downregulation of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase and VEGF expression in rat NAFLD model, thus, in turn, impedes obesity, NAFLD, NASH, and fibrosis. Additionally, the extract administration exerted antioxidant activity *via* increasing GSH, SOD, and CAT activities as well as reduction of MDA and NO levels.[62]

In another study, Sakran et al.[63] isolated 5,6-dimethoxy-2',3'-methylenedioxy-7-C-β-D-gluco-pyranosyl isoflavone (a new isoflavonoid) from *L. sativum* seeds. They showed that this new isoflavonoid have a potential to diminish the hepatotoxicity induced by paracetamol in adult Sprague Dawley male rats. They proposed that hepatoprotective effect depends on enhancing TAC and normalizing level of liver enzymes including GSH, SOD, GPX, CAT, and GST.[63]

Antidiabetic activity

Hyperglycemia (high blood sugar) causes long-term complications in affected people. Untreated hyperglycemia results in renal failure, diabetic cataract, elevated risk of cardiovascular diseases, and excessive generation of free radicals. Numerous lines of evidence suggested ethnomedicinal plants in order to ameliorate the disease and lessen the side effects of synthetic drugs.[64] In line with this, Attia et al.[64] demonstrated that *L. sativum* seeds methanolic extract, reduced blood sugar and reversed all biochemical and histological complication of alloxan-induced diabetes in rat model.[64] In another study, hypoglycaemic activity of aqueous extract of *L. sativum* seeds was examined in streptozotocin (STZ)-induced diabetic rat model. Their results displayed a significant blood glucose level reduction without any substantial alternation in basal plasma insulin concentration, which supports the concept that its hypoglycaemic activity may occur independent of insulin secretion.[65] Another study performed in hypercholesterolemic albino male rats revealed that *L. sativum* seed extract improved lipid profile [decrease in cholesterol, TGs, LDL, and increase in high density lipoprotein cholesterol (HDL)] and markedly diminished blood glucose in comparison to the control group.[66] Furthermore, Eddouks and Maghrani[67] designed a study to investigate the mechanisms underlying the hypoglycaemic activity of *L. sativum* in STZ-induced diabetic rats. Their results showed that administration of the aqueous extract decreased blood glucose, increased glycosuria, and normalized glycaemia through prevention of renal glucose reabsorption that is independent of any alternations in insulin secretion.[67] *L. sativum* seed powder administration in alloxan-induced diabetic male Wistar rats decreased fasting blood glucose levels, glycosylated haemoglobin (Hb A1C %), TG, lipid profile [TC and lipoprotein fractions (LDLc and VLDLc)]. The extract treatment also elevated the HDLc levels significantly. Additionally, a marked decrease in TBARS levels and increase in GSH and antioxidant enzyme activity was detected in extract treated rats.[68] In a more recent study, Ullah et al.[69] revealed that light (as great abiotic elicitor) play a critical role in biosynthesis of herbal metabolites. Data from their research illustrated that, callus cultures of *L. sativum* under white light exerted maximum level of phenolic profile, antidiabetic, and antioxidant properties compared to other conditions *in vitro*.[69] It was also marked by L'hadj et al.[70] that *L. sativum* flavonoid-rich extract had potential hypoglycemic, hypolipidemic, anti-inflammatory, cytoprotective, and antidiabetic properties in Wistar rats *via* enhancing dyslipidemia, insulin sensitivity, inflammation, and pancreas β-cell integrity.[70]

Impacts on reproductive health

Cumulative evidence proposed the capability of herbal medicine in improving reproductive dysfunction or fertility due to their phytochemicals.[71] In a recent study in doe rabbit model, *L. sativum* oil increased the level of the reproductive hormones, while improved antioxidant status and reproductive performance (receptivity, conception rate, and litter size).[71] Moreover, Kamani et al.[72] experimented efficacy of ethanolic extract of *L. sativum* seed on histopathological alternations

of epididymis in STZ-induced diabetic adult male Wistar rats. Their findings exhibited the improved epithelium height as well as reduction of interstitial volume density, fibromuscular thickness, volume density of epithelium through preventing oxidative stress, which, in turn, demonstrated the extract's protective effect on reproductive system.[72] Recently, Asl et al.[73] revealed that co-administration of coenzyme Q10 (CoQ10) and *L. sativum* markedly enhanced the hypothalamic-pituitary-gonadal axis activity and ameliorated the reproductive functions in adult male mice. Co-administration of CoQ10 and *L. sativum* resulted in elevation of all features of sexual behaviors and serum testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels as well as sperm viability and motility.[73] Another animal study was performed to examine the impact of aqueous extract of *L. sativum* on fertility criteria in male mice. Findings of this study displayed that extract-treated mice had higher levels of FSH and testosterone. Overall, all the infertility parameters improved in the hyperprolactenimic animals treated with the extract. Histological analysis of the testis in the extract-treated mice exhibited normal status of seminiferous tubule with high number of sperms.[74] Imade and colleagues investigated the effects of *L. sativum* seeds on the male reproductive functions in rabbit bucks. Rabbits fed with *L. sativum* seeds significantly elevated plasma LH concentrations without any significant difference in testosterone levels. Motility and live sperm percentage were significantly decreased in *L. sativum* seed-treated rabbits. Besides, sperm abnormality percent was increased significantly in *L. sativum* seed-treated rabbits dose-dependently. Taken all together, in case of high amount consumption, toxic effects of *L. sativum* seed on sperm quality and testis in rabbit bucks were observed.[75] In another study, *L. sativum* elevated the concentrations of estrogen, progesterone, LH, FSH, and free testosterone hormones in female rabbits. Besides, significant augmentation of sexual receptivity, conception rate, gestation length, litter size, and body weight at birth in extract treated groups were detected. Overall, *L. sativum* elevated reproductive hormone level and performance *in vivo*.[71]

Osteoprotective activity

Osteoporosis is a progressive "skeletal disorder characterized *via* low bone mass, micro-architectural deterioration of bone tissue leading to increased risk of bone fragility and fracture risk".[53] In traditional medicine, *L. sativum* seeds have been proposed to have potential in healing bone fractures.[53] In light of this record, recently, the fracture healing potential of the methanolic and aqueous extracts of *L. sativum* seeds in rats was experimented by Dixit et al.[76] Biochemical and radiological analysis revealed that the methanolic extract markedly led to callus formation. It has been reported that ibuprofen exerted toxic effects on the osteocytes in bone tissue, whereas various concentrations of the aqueous extract of *L. sativum* seeds inhibited the effects of ibuprofen in male albino rats.[77] Administration of teriparatide (a recombinant parathyroid hormone utilized as antiosteoporotic therapy) and *L. sativum* seeds ameliorated biochemical, histological, and morphometric bone alternations induced by glucocorticoids in male guinea pigs through osteocytes apoptosis reduction as well as osteoclasts elevation.[78] Abdallah et al.[79] examined osteoprotective effect of *L. sativum* extract in an ovariectomized rat model. Their findings demonstrated that the extract improved bone weight, bone formation biomarkers (LDH and osteocalcin) levels, and free radical scavenging activity (through enhancing SOD and GPX activities). Furthermore, oral administration of the extract results in increase of the bone resorption markers [*e.g.* carboxyterminal telopeptide, type I and, tartrate-resistant acid phosphatase (TRAP)] and regulation of receptor activator of nuclear factor kappa-B ligand/osteoprotegerin expression. Taken all together, they suggested that presence of glucosinolates, lignans, coumarins, phenolic acids, and alkaloids leads to the aforementioned antiosteoporotic effects synergistically.[79] In another research, the synergistic antiosteoporotic activity of *L. sativum* and alendronate in glucocorticoid-induced osteoporosis was evaluated by methylprednisolone injection in adult female rats. Their findings revealed that *L. sativum* alone and/or in combination with alendronate treatments, markedly diminished serum TRAP and improved bone-ALP, phosphorus, calcium, and bone architecture (through increasing trabecular area or bone marrow area percentage in the proximal femoral epiphysis).[80] Further, Juma[81] revealed that *L. sativum* seeds significantly improved fractures healing in New Zealand white rabbits, which documented *via* direct measurements of callus formation in millimeters at the longitudinal medial and longitudinal lateral and circumferential areas. Administration of *L. sativum* seed powder to the rabbits with bone fractures demonstrated a significant increase in bone markers, *e.g.* osteopontin and vitamin D, parathormone, and lactoferrin levels as well as reduction in serum levels of osteocalcin, when compared to the untreated group.[82]

Anti-asthmatic activity

Bronchial asthma is a chronic inflammatory disease of airways of the lung, characterized by hyper-reactivity of the airways to various stimuli. Its clinical manifestations include paroxysmal dyspnea, wheezing cough, and a sense of thoracic constriction. From ancient times, the efficacy of the natural remedies in healing various diseases including bronchial asthma, hiccups, cough, *etc.* has been elucidated.[83] Nevertheless, there is lack of scientific studies, which investigated the efficacy of *L. sativum* in bronchial asthma treatment. For that reason, Paranjape and Mehta[83] carried out a clinical trial to assess the efficiency and safety of *L. sativum* in bronchial asthma affected patients. After 4 weeks of treatment with *L. sativum* seed powder, substantial improvement in several parameters of pulmonary functions, clinical symptoms, and severity of asthmatic attacks without any adverse reaction were observed in asthmatic subjects.[83] In another study carried out on guinea pigs, the bronchodilatory effect of the ethanolic extract of *L. sativum* seeds was investigated in histamine and acetylcholine induced acute bronchospasm. Data from their study showed that the extract, markedly protected guinea pigs against bronchospasm in comparison to the ketotifen and atropine sulphate (as reference drugs).[84] Additionally, Rehman et al.[85] indicated that a combination of anticholinergic, Ca^{2+} antagonist,

and PDE inhibitory pathways were responsible for *L. sativum*'s bronchodilatory activity.

Neuroprotective effects

A few studies stated potential neuroprotective activity of *L. sativum*. In this regard, El-Ghazouly et al.[86] assessed neuroprotective effects of *L. sativum* aqueous extract on the cerebellum of adult male albino rats. Methotrexate exerted adverse effects on cerebellum by reducing the number of Purkinje cells with significant reduction of Nissl's granules. However, in methotrexate and *L. sativum* aqueous extract administrated rats approximately normal histological appearance of Purkinje cells with less vacuolated cytoplasm was observed, which was validated by a substantial rise in the Purkinje cells number, significant diminution in caspase-3 positive cells and in GFAP immunostaining.[86] Moreover, neuropharmacological impact of the alkaloid of *L. sativum* was evaluated in Swiss albino mice and Wistar albino rats. The results of this study elucidated sedative, anxiolytic, myorelaxant, and analgesic effects of *L. sativum* alkaloid through diminished locomotor activity and motor coordination, and increased preference to plus maze open arm.[87] Al-Dbass et al.[88] examined the potential beneficial impact of *L. sativum* seed extract against glutamate excitotoxicity-induced retinal ganglion cell degeneration, which results in severe blindness. The extract enhanced the cell viability in retinal ganglion cells after exposure to the high concentrations of the glutamate. Thus, they deduced that *L. sativum* seed extracts might exert effective anti-excitotoxic and antioxidant activity in various neurological disorders.[88]

Other medical effects

Several studies have evaluated various beneficial effects of *L. sativum* extract in animal models. The ethanolic extract of *L. sativum* seeds significantly prohibited carrageenan-induced paw edema and reduced the yeast-induced hyperpyrexia in mice models and exerted anti-inflammatory and antipyretic effects, respectively. The coagulation studies demonstrated elevated levels of fibrinogen and negligible reduction in prothrombin time, which, in turn, validated the coagulant activity of the extract.[89] The ethanolic extract of *L. sativum* seeds exerted significant anti-inflammatory activity in carrageenan-induced paw edema in mice through improving biomarkers of inflammation (serum albumin, C-reactive protein, and plasma fibrinogen) in comparison to the control group.[90]

Alkharfy et al.[91] examined drug-herb interactions and proposed that simultaneous consumption of herbs significantly changed the phenytoin (an anticonvulsant drug) disposition in a dog model. In a follow-up study, the methanolic extract of *L. sativum* seed exerted genoprotective effect by inhibition of DNA aberrations in somatic and germ cells of mice dose-dependently. They proposed that the flavonoidal content and antioxidant activity may be responsible for this beneficial properties.[92] Another *in vivo* study performed to evaluate the safety of *L. sativum* seeds powder in adult Wistar rats. Administration of *L. sativum* powder considered non-toxic and safe because of insignificant changes in food intake, gain in body weight, relative weight of organs, *e.g.* liver, lungs, kidney, spleen, brain, adrenals, gonads, and heart, hematological parameters [including: red blood cells (RBC), white blood cells (WBC), hemoglobin, mean corpuscular hemoglobin (MCH), and MCH concentration], macroscopic and microscopic changes in vital organs, in the experimental group in comparison to the control group.[93]

In a recent study, for the first time, the effects of the aqueous *L. sativum* seed extract on the immune system and general health were reported in mice model. The results demonstrated that the extract caused a boost in immune system through WBC types, RBC, and platelet counts as well as mean hemoglobin concentration, mean total body weight gains, and weights of the organs.[94] Moreover, addition of *L. sativum* seeds to the diet of rats for the first 3 weeks, resulted in elevated mean body weights and body weight gains.[95] In a study performed by Kaur and Sharma[96], it has been elucidated that the supplementation of *L. sativum* seeds (for 2 months) moderately increased the haemoglobin (g/dL) levels among anemic adolescent girls possibly because of iron content.[96]

Diwakar et al.[97] investigated the modulatory effect of ALA-rich *L. sativum* seed oil on lipid composition, spleen lymphocyte proliferation and inflammatory mediator production in rat model. Data from their research illustrated that the extract modulates inflammatory mediators (NO, leukotriene B4), consequently alleviates inflammatory responses.[97]

CONCLUSION

Various herbs and their extracts have gained substantial interest since they encompass diverse phytochemicals which represents numerous health-promoting activities. Using different parts of *L. sativum,* several pre-clinical studies demonstrated their potential in alleviating different disorders and improving health (*e.g.* antimicrobial, antioxidant, anticancer, antidiabetic, anti-asthmatic, and many other protective activities). Hence, *L. sativum* has been considered as an attractive alternative over the conventional therapeutics due to their nutritional values, and less or no adverse effects. However, further comprehensive studies are required to define molecular mechanisms underlying certain health-promoting properties and provide more convincing evidence for the efficacy of *L. sativum*.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Y.H., Y.R.S., Design: Y.H., Y.R.S., Data Collection or Processing: Y.H., T.Ö., Analysis or Interpretation: Y.H., T.Ö., Literature Search: Y.H., Y.R.S., T.Ö., Writing: Y.H., Y.R.S., T.Ö.

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