Content

Original Article

Microbial Growth Inhibitory Activities of Extracts from the Peels, Juice Vesicles, and Seeds of *Citrus paradisi*

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Abstract

Introduction: *Citrus paradisi* is abundant in insoluble pectin fibre, rich in vitamin C and phytochemicals. With the increasing resistance of common clinically-important pathogens (especially bacteria) to antibiotics today, it brings the focus to antimicrobial properties of plant extracts containing bioactive phytochemical compounds which can potentially kill or inhibit the growth of microorganisms. **Objective:** This study aims to measure the antimicrobial effects of methanolic extracts of *C. paradisi* peels, juice vesicles, and seeds. **Materials and methods:** These samples were procured fresh and processed prior to antimicrobial assays, including disk-diffusion assays and minimum inhibitory concentration tests. In addition, independent *t*-tests and 1-way ANOVA were integrated to study the significance of microbial growth inhibition. **Results:** *B. subtilis* was found possessing the highest sensitivity towards all extracts, especially to juice vesicles and seed extracts. Other microorganisms (eg. *Staphylococcus aureus* and *Escherichia coli*) exhibited a moderate level of tolerance or sensitivity towards different extracts. **Conclusion:** To our knowledge, this is the first study reporting the antimicrobial effects of *C. paradisi* juice vesicles. We have also highlighted the needs in identifying and investigating the phytochemical(s) which are effective in inhibiting microbial growth.

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Introduction

In view of the increasing concern regarding antibiotic-resistant bacterial strains worldwide, plants have been serving as a good source of medicinal agents[1], including antimicrobial agents. *Citrus paradisi*, conventionally known as grapefruit, belongs to the family of Rutaceae. It is generally believed to have originated from a natural cross-hybridisation between *Citrus grandis*

(pomelo) and *Citrus sinensis* (orange). *C. paradisi* usually presents a yellow outer peel, with their pulps in either red or pink which indicate the presence of lycopene[2].

There are various health benefits of *C. paradisi*. A rich amount of soluble fibre - pectin has been found in the pulps. Several studies have confirmed the medicinal value of pectin in preventing prostate and colon cancer[3,4]. On the other hand, a membranous content found inside the citrus fruit's endocarp - juice vesicle is filled with lipids which improve the scent or aroma of citrus fruits[5]. On the other hand, extracts from the seeds of *C. paradisi* have been shown to carry antimicrobial properties[6,7]. Despite these discoveries, the antimicrobial properties of extracts from *C. paradisi* peels, fruits (especially juice vesicles), and seeds against human pathogens remained elusive.

Despite its health benefits, the juice of *C. paradisi* has exhibited juice-drug interaction as well. These properties have affected drug bioavailability in the recipients. In 2019, it was reported that the juice of *C. paradisi* inhibited the intestinal CYP3A4 enzyme thus causing the augmentation or decrement of drug bioavailability[8]. Another organic compound that may interfere with the bioactive compound of *C. paradisi* juice is multidrug resistance protein 1 (MDR1), which can be found in the human intestinal epithelium acting as drug efflux pumps[9]. Also, the juice of *C. paradisi* inhibits the organic anion transporting polypeptides (OATPs), which serve as drug transporters across the cell membrane of kidney and liver cells[10]. In addition, several drugs which exhibit juice-drug interaction with *C. paradisi* were also reported earlier[11–13].

In order to avoid juice-drug interaction while applying antimicrobial properties of *C. paradisi* in clinical / non-clinical settings, further investigation on antimicrobial properties of Citrus fruits is needed. Numerous studies are available for antimicrobial properties of *Citrus sinensis* (oranges) and *Citrus limon* (lemons).

In 2018, a study reported the antimicrobial activity of *C. sinensis* peel organic solvents and aqueous extracts. The results proved that the extracts of *C. sinensis* peel possessed a significant antimicrobial activity against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans*[14]. Other studies investigated the antibacterial activity of *C. sinensis* peel and juice extracts. Their results showed a remarkable antibacterial activity of the extracts against *S. aureus, E. coli* and *P. aeruginosa*[15,16]. On the other hand, several research groups have reported the antimicrobial characteristics of *C. limon* methanolic

or ethanolic extracts against important microbial pathogens, including *E. coli*, *C. albicans* and *S. aureus*[17,18].

Compared to *C. sinensis* and *C. limon*, the antimicrobial properties of *C. paradisi* extracts remained elusive. Although data were available in 2001 and 2004, Negi and Jayaprakasha reported the antimicrobial properties of *C. paradisi* peel extracts[19], while Cvetnić and Vladimir-Knezević reported using its seed extracts otherwise[20]. Therefore, this study was designed to update the research gaps pertaining to the antimicrobial properties of *C. paradisi* peel and seed extracts under the same experimental settings. In addition, we have included an additional sample - juice vesicle's extract of *C. paradisi* in this study. To our knowledge, this is the first research study focusing on the antimicrobial effects of *C. paradisi* extracts from its juice vesicles.

Materials and Methods

Procurement of samples: *C. paradisi* fruits were purchased from local grocery stores located in Klang Valley, Malaysia.

Preparation of *C. paradisi* extracts: The peels, juice vesicles, and seeds of *C. paradisi* were dried in an oven (60℃, 72 hours). The dried samples were ground into powders using pestle and mortar. Then, these powders were mixed with methanol (99%) until the samples were fully immersed. All bottles were kept in an incubator (45℃, 72 hours), with swirling using magnetic stirrers. After 72 hours, all mixtures were filtered, with filtrates placed in bottles half immersed in a water bath (50℃) to evaporate the excess methanol. The filtrates were then transferred into an oven (60℃) until the methanol was dried completely. Next, the weight of peel, juice vesicle, and seed extracts were measured and recorded.

Reconstitution of extracts: To reconstitute the dried powders, methanol (99%) was added to dissolve the dried extracts. The mixtures were sonicated to ease the process of reconstitution. The reconstituted extracts were kept in -20℃ until used.

Antimicrobial assays: All experimental procedures were conducted under an aseptic environment. The bacterial strains used in this study were *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. Bacterial cultures were maintained on nutrient agars. Plate cultures were transferred to nutrient broth (Oxoid, CM0001) to 0.5 McFarland standard, with 50 µL of the nutrient broth culture inoculated onto a fresh nutrient agar. Inoculum was spread evenly using a sterile L-shape glass rod.

Filter paper disks were prepared, autoclaved, and dried prior to this procedure. Sterile filter paper disks were soaked thoroughly in the prepared extracts, and air-dried in a biosafety cabinet. Dried filter paper disks were placed on the surface of inoculated nutrient agars. Then, the plates were placed in an incubator (37℃, 18 hours). After that, the diameter (in milimeter, mm) of inhibition zones were measured and recorded.

Determination of minimum inhibitory concentrations (MIC): MIC tests were carried out using serial dilution method. Since *C. paradisi* juice vesicles showed a significant microbial growth inhibitory effect, MIC was conducted only to juice vesicle extracts. First, a serial dilution of bacterial cultures were prepared in sterile 1.5 mL tubes (11 tubes ranged from 1580 mg/mL to 0 mg/mL). Bacterial cultures of *S. aureus*, *E. coli*, and *B. subtilis* were prepared to 0.5 McFarland standard, with 10 µL of each culture inoculated into all mixtures with different concentrations of *C. paradisi* juice vesicle extract. All tubes were placed in an incubator (37 ℃, 18 hours). After that, the MIC of each extract was determined by observing the turbidity of the solutions, and confirmed by inoculation of each sample onto fresh nutrient agar plates.

Statistical analyses: Statistical analyses were conducted to determine the significance of difference between the type of *C. paradisi* extract (ie. peels, juice vesicles, seeds) and their antimicrobial effect against different microorganisms (ie. *E. coli, S. aureus, B. subtilis*). Analyses were conducted using Statistical Package for the Social Sciences (SPSS®, IBM®), version 23. One-way Analysis of variance (1-way ANOVA) was measured with *P* value of <0.05 considered as significant data.

Ethics approval:This project was conducted with approval by SEGi University Ethics Committee (Ethics number: SEGiEC/SR/FOP/10/2020-2021).

Results

C. paradisi extractions: Methanolic extracts of *C. paradisi* were measured and documented in Table 1 below. Overall, the yield from juice vesicles was higher compared to peels and seeds (Table 1).

Antimicrobial assays (disk diffusion assays): Antimicrobial properties of each extract were determined *via* disk diffusion assays. The microbial inhibitory effects of each sample were measured and presented in Figure 1.

Determination of minimum inhibitory concentrations (MIC): The presence of live microorganisms (either *E. coli, S. aureus,* or *B. subtilis*) was detected by viewing the turbidity

of each culture tube. This was complemented by observing the presence of the respective microorganisms on nutrient agars (Table 2).

The minimum inhibitory effect of *C. paradisi* juice vesicle extract was determined at between 790 - 1580 mg/mL, 395 - 790 mg/mL, and 197.50 - 395 mg/mL for *E. coli*, *S. aureus* and *B. subtilis*, respectively (Table 2).

Statistical analyses: The *P* values of One-way Analysis of Variance (1-way ANOVA) are indicated in Table 3. The significance of differences between antimicrobial effects between the parts of *C. paradisi* were measured at 0.256, 0.075, and 0.016 against *E. coli*, *S. aureus*, and *B. subtilis*, respectively.

Table 1: Concentrations of *C. paradisi* extractions.

Parts of *C. paradisi*	Concentrations of extracted sample after reconstitution (g/mL)	
	1st extraction	2nd extraction
Peels	0.3577	0.4030
Juice vesicles	1.0746	1.5801
Seeds	0.1294	0.1805

Table 2: The presence of microorganisms detected in each culture tube with serial dilution of *C. paradisi* juice vesicle extract.

Culture tube	Final concentration (mg/mL) of *C. paradisi* juice vesicle extract	Presence of microorganism		
		E. coli	*S. aureus*	*B. subtilis*
1st	1580.00	**No**	**No**	**No**
2nd	790.00	Yes	**No**	**No**

3rd	395.00	Yes	Yes	**No**
4th	197.50	Yes	Yes	Yes
5th	98.75	Yes	Yes	Yes
6th	49.38	Yes	Yes	Yes
7th	24.69	Yes	Yes	Yes
8th	12.34	Yes	Yes	Yes
9th	6.17	Yes	Yes	Yes
10th	3.08	Yes	Yes	Yes
11th	0.00	Yes	Yes	Yes

Table 3: 1-way ANOVA analyses between the types of *C. paradisi* extracts and their antimicrobial effects.

Type of microorganism	Source of variation	*P*-value
E. coli	Between Groups	0.256
S. aureus	Between Groups	0.075
B. subtilis	Between Groups	**0.016***

* Significant value(s)

Figures

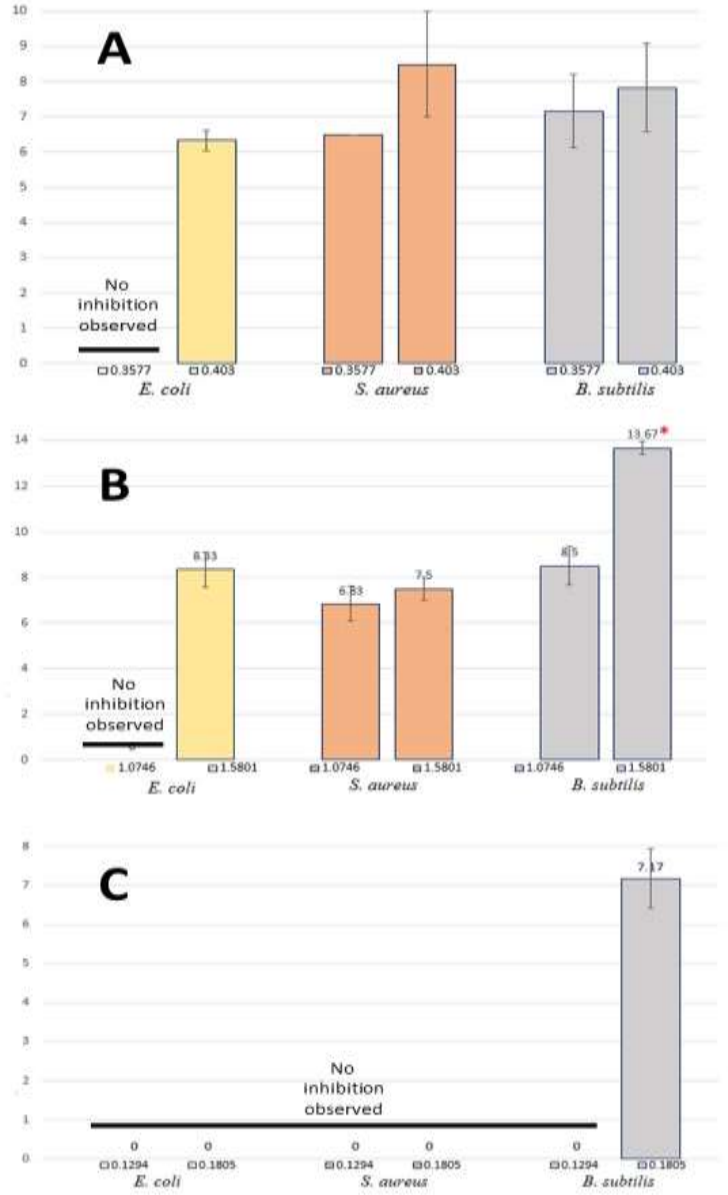

Figure 1: The average diameter of zone of inhibition (mm) of *E. coli*, *S. aureus*, and *B. subtilis* against different concentrations of extracts after reconstitution. (Y-axis) Average diameter of inhibition zones (mm); (X-axis) Different strains of microorganisms inhibited by the respective reconstituted methanolic extracts; **(A)** Peel extracts of *C. paradisi*; **(B)** Juice vesicle extracts of *C. paradisi*; **(C)** Seed extracts of *C. paradisi*. Asterisk indicates a significant value ($P = 0.0005$) found in the growth inhibition of *B. subtilis* by juice vesicle extracts.

Discussions

Despite previously reported studies, this study has highlighted several variations on antimicrobial effects of peels, juice vesicles, and seeds of *C. paradisi*. These variations can be derived from the differences in chemical constituents of polyphenols from various parts of *C. paradisi*, as reported in previous studies[21–23].

In general, *E. coli* exhibited a higher resistance against methanolic extracts of all parts of *C. paradisi*. This could be ascribed to the differences of cell wall components between Gram-positive and Gram-negative bacteria. The outer lipopolysaccharide layer and a peptidoglycan-containing periplasmic space of Gram-negative bacteria have been proven to confer additional protection to certain microorganisms[24,25]. In addition, some enzymes found in the periplasmic space of Gram-negative bacteria have been reported to prevent the entry of foreign molecules into the cytoplasm[26].

The peel methanolic extracts of *C. paradisi* were prepared at 2 different concentrations (ie. 0.3577 g/mL and 0.4030 g/mL). Other than the sample with 0.3577 g/mL against *E. coli*, both concentrations exhibited microbial inhibitory effects towards *E. coli*, *S. aureus*, and *B. subtilis* (Figure 1). For *E. coli*, the inhibitory effect increased significantly when the concentration increased from 0.3577 g/mL to 0.4030 g/mL ($P = 0.0007$). This result was consistent with research findings previously reported[6,27,28]. Similar trends were reported for *S. aureus* and *B. subtilis*. The findings of this study described the microbial inhibitory effects of *C. paradisi* peel extracts against *S. aureus*, and these findings are also in accordance with several previous studies[6,29]. Similarly, for *B. subtilis*, microbial inhibitory effects were also reported previously with different lengths of inhibitory zones[6,27]. Upon investigations, the differences in the zone of inhibition can be due to the variables during plant extract preparations and the status of microorganisms during inoculations. While most of the reported studies employed essential oils at high concentration, our study extracted pure methanolic extracts from fresh *C. paradisi*, which were used in sample testing without a long delay.

The *C. paradisi* juice vesicle methanolic extracts exhibited a similar inhibitory pattern against *E. coli* and *S. aureus* (Figure 1B). A significant improvement in growth inhibitory effect was observed in *E. coli* when the extracts of juice vesicles increased from 1.0746 g/mL to 1.5801 g/mL ($P = 0.003$). The same pattern was also observed in *B. bacillus* ($P < 0.0005$) (Figure 1B, marked with asterisk). To our knowledge, this is the first study reporting the microbial growth inhibitory effect of *C. paradisi* juice vesicle extracts.

Although the seed extract of *C. paradisi* was reported not to hold any antimicrobial effect against *E. coli*, *S. aureus*, and *B. subtilis*[30], our results suggest that the extracts possess microbial growth inhibitory effect against *B. subtilis* at concentration of 0.1805 g/mL (Figure 1C). Compared to a study by Sahlan *et al.*, our seed extract does not exhibit a strong inhibitory effect towards *B. subtilis*[31]. This can be explained by the different parts of *C. paradisi* used in extract preparation, whereby Sahlan *et al.* employed a mixture of *C. paradisi* seeds and pulps, while this study presented the inhibitory effect of extracts purely prepared from seeds only. Similar to juice vesicle extracts, the findings on seed extracts have highlighted the need to further investigate the phytochemical compounds which play their antimicrobial roles.

The significant increase ($P = 0.0005$) in microbial growth inhibitory effect towards *B. subtilis* by juice vesicle extracts has drawn our interest in its antimicrobial activities (Figure 1B). Therefore, its minimum inhibitory concentration (MIC) against different microorganisms was tested, with results tabulated in Table 2. Limited studies are available to deduce the MIC of *C. paradisi* juice vesicle extracts. Although some researchers reported a much lower MIC of *C. paradisi* extract towards *E. coli*, *S. aureus* and/or *B. subtilis*[20], these studies analysed phytochemical activities of *C. paradisi* seeds, pulps, or citrus juice concentrates. Our findings tabulated in Table 2 show the same inhibitory patterns as disk-diffusion assay (Figure 1B), whereby *B. subtilis* exhibited the highest sensitivity towards the juice vesicle extract of *C. paradisi*. This phenomenon indicated the presence of specific phytocompounds which can effectively suppress the growth of *B. subtilis*, which can be lethal to vertebrate animals once being introduced into the animals[32].

One-way Analysis of variance (1-way ANOVA) analyses were conducted to measure the significance of difference between the type of *C. paradisi* extract (ie. peels, juice vesicles, seeds) and their antimicrobial effect against different species of microorganisms (ie. *E. coli*, *S. aureus*, *B. subtilis*). As shown in Table 3, our results have clearly indicated that *B. subtilis* exhibited a significant difference in sensitivity towards different types of extract from *C. paradisi*. This finding has again highlighted the needs in exploring and identifying the phytochemical compounds in the seeds and juice vesicles of *C. paradisi* that exhibit the antimicrobial properties.

Conclusion

This study has reported the antimicrobial effect of *C. paradisi* extracts from its peels, juice vesicles, and seeds against both Gram-positive and Gram-negative bacteria (ie. *E. coli*, *S.*

aureus, *B. subtilis*), using common methodologies including disk-diffusion assays, minimum inhibitory effect (MIC) tests, and some integrations of statistical analyses (independent *t*-test, 1-way ANOVA). The results indicated that the well-studied Gram-positive bacteria - *B. subtilis* possessed a high sensitivity towards *C. paradisi* extracts, especially those prepared from juice vesicles and seeds. MIC tests have also highlighted the research needs in identifying the phytochemical compounds in these samples for characterisation studies. To our knowledge, this is the first study elucidating the antimicrobial effect of dried *C. paradisi* juice vesicles, instead of the while pulp or extracted fruit juice.

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Original Research

Knowledge, Attitude, and Practices of Dental Undergraduate Students on Infection Control In Dental Clinic During COVID-19 Pandemic - A Cross Sectional Study.

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Abstract:

Objectives: COVID-19 infection transmits through droplets in air. Dental setting can act as potential site for cross-contamination, and hence, calls for strict infection control protocols during dental treatment. Hence, this study was undertaken to assess the knowledge, attitude and practices of dental undergraduate students on infection control during COVID-19 pandemic, so that suitable measures can be undertaken to educate them. **Methods:** A cross-sectional survey was undertaken among 135 clinical year students of Faculty of Dentistry, SEGi University, Malaysia. A questionnaire containing 37 close ended questions pertaining to infection control during the COVID-19 pandemic, was developed and validated at the faculty. The survey was electronically disseminated through google forms. Data was analysed using SPSS version 22. Descriptive statistics and chi-square test were used. $p<0.05$ was considered statistically significant. **Results:** The response rate was 97%. All of our students exhibited adequate knowledge pertaining to COVID-19 infection. About 95% of the students showed positive attitude towards patient triage, use of personal protective equipment, social distancing in clinic, use of high vacuum suction for aerosols. However, about 50% felt stressed to treat patients during this pandemic and only 15% were willing to treat patients. Regarding practices,80% followed correct order of donning, however only 25% did the doffing in correct manner. **Conclusion:** Though our students exhibited good knowledge, there is a need to educate and reinforce right attitude and correct practices pertaining to infection control, for a safe and effective clinical practice in times of such pandemic. The faculty has developed strategies for the same.

Key words: COVID-19, Infection control, Dental clinic, Dental students

Introduction:

COVID-19 is an infectious disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). [1] The World Health Organization (WHO) on March 11, 2020, declared the novel coronavirus (COVID-19) outbreak a global pandemic. [2] The measures taken to stop the spread of this disease have included isolation, contact tracing and quarantine, social distancing, hygiene measures, and lockdown. [3-5] The transmissibility of COVID-19 is higher than that of other similar respiratory diseases such as severe acute respiratory syndrome (SARS-CoV) and the Middle East respiratory syndrome coronaviruses (MERS-CoV). [6]

Droplets and aerosols are known to be two major mechanisms of its spread. [1] Droplets released after exhalation, coughing, or sneeze cause airborne contamination. [7] Also, contact with contaminated surfaces and with the eyes, nose, or mouth causes direct contamination. [8]Particles from droplets remain in suspension for a length of time that depends on their size, settling velocity, relative humidity, and airflow in which droplets can spread up to 1 m. [9] In addition, it has been reported that even in the absence of clinical symptoms, the virus can still spread. [10,11] Therefore, health care workers, including dentists, are at high risk for acquiring and transmitting infection within their work environment due to close contact with patients and the instruments they use, such as dental handpieces and scalers that spread droplets, and aerosols of blood and saliva. [7] This calls for a serious control of the spread of infectious disease such COVID-19 in dentistry.

As a consequence of this pandemic, caution in handling patients in the waiting room and during treatment by using personal protective equipment (PPE) has been increased. The interim guidelines of the CDC, and the American Dental Association (ADA) have provided guidelines about the measures for infection control during the new normal period, including required instruments and best practices to prevent the infection transmission. These guidelines include careful patient evaluation and appropriate hand hygiene, donning Personal Protective Equipment (PPE) such as protective eyewear, surgical masks, gloves, caps, face shields, and protective outwear etc. [12-14]

The high risk of COVID-19 infection among dental staff emphasizes their equally important role in preventing and controlling its transmission. Although dental students have learned how to deal with infectious diseases and take preventive measures, the need to be aware of a new protocol adjusted for COVID-19 is of great importance, and this depends on their level of

awareness of the disease. Hence, this study aimed to assess knowledge, perceptions, and attitudes regarding COVID-19 and infection control measures against it among dental students.

Methodology:

This cross-sectional descriptive study was carried out in December 2020, at Faculty of Dentistry, SEGi University, Malaysia. The study involved clinical year students of Year 3,4 and 5. Ethics approval was obtained from institutional ethics committee (SEGiEC/SR/FOD/36/2020-2021). The permissions of the students were obtained before sending the questionnaires. Students who did not want to be part of the survey were excluded.

A questionnaire on the COVID-19 pandemic was prepared by a team of specialists, which included its impacts on the field of dentistry and the protocols and guidelines of infection control regarding the novel coronavirus. The questionnaire was reviewed by three dental specialists experts in infection control and to assess the content and face validity. They checked the questions to ensure they were associated with the topic and effectively captured the variables and added more related questions if necessary. A pilot study was conducted where in the questionnaire was given to a random sample of 10 respondents (graduated students) to assess reliability. The internal consistency of questions was checked by Cronbach's alpha and the value was 0.83. Finally, a 34-item questionnaire was prepared in google forms. The questionnaire was sent to all the dental students of the clinical phase. The questionnaire consisted of two sections, including the demographic part and a section associated with the knowledge and attitude. The demographic data included the following three

Items: age, gender and year of study. The second part of the questionnaire consisted of 31 items : 10 questions pertaining to knowledge such as awareness of COVID-19, the etiology of COVID -19, the mode of transmission; 14 pertaining to attitude such as willingness to treat COVID-19 patients, confidence to treat COVID-19 patients; 10 pertaining to practices such as proper PPE performed before treating patients, correct order of donning and doffing, practice of social distancing. Survey was then disseminated electronically using google forms via email and whats app to the Year 3,4 and 5. Partially-filled questionnaires were excluded from the study.

Data Analysis

Survey data from the google forms was downloaded as Excel , analysed using SPSS version 22 .Descriptive statistics like percentage, mean and standard deviations were used for data interpretation.

Results

The survey was sent to 132 students and the response rate was100%. The students were aged between 21 to 28 years with the mean age of 23.7+ 0.3 years. One third of the participants (33.3%) were males and two thirds were females (66.6%). The participants consisted of Year 3(33.3%), Year 4(40.2%) and Year 5(35%) students.

The results have been presented as pertaining to Knowledge, Attitude and Practices.

A. Knowledge:

Table 1 : knowledge of the participants pertaining to the transmission of the virus in the dental clinic.

Questions	**Yes(%)**	**No(%)**	**Maybe(%)**
Had you heard of COVID-19 disease?	100	0	0
Were you aware it was caused by a virus?	100	0	0
Are you aware that the way of transmission is through air droplets?	100	0	0
Are you aware of all of the most common symptoms seen in a COVID-19 positive patient?	90.2	9.9	0
Do you know that the elderly above 60 years are most affected from the COVID-19 virus?	92.4	6	0
Do you know that people with co-morbidities have higher mortality rates when having the COVID-19 virus?	91.7	0	8.3
Do you believe that it is safe to treat patients in dental clinics?	40.9	25.8	33.3
Are you aware that aerosol generating procedures can spread the virus?	97	2.3	0.8
Do you believe that masks provide 100% protection when facing patients with the virus?	75.8	9.8	14.4

Are you aware of the proper personal protective equipment?	100	0	0

Table 1 showed the knowledge pertaining to COVID-19 transmission and control. 100%of our students had heard about the COVID, the agent causing it and that COVID-19 spreads through droplets in the air. More than 90% of the students were aware of the signs and symptoms, and that the virus had a stronger detrimental health effects on elderly above the age of 60 years and patients with co-morbidities. However, only 40% of our participants believed that it was safe to treat patients during this pandemic . More than 95% of our participants believed that aerosol generating procedures could spread the virus. About 76% felt that masks provided 100% protection when facing patients with the virus . All our participants were aware on the importance on using personal protective equipment when treating the patients.

B. Attitude

Table 2 : Attitude of the participants towards infection control in dental clinic during the pandemic.

Questions	**Yes (%)**	**No (%)**	**Maybe (%)**
Do you agree on the importance of taking patient's history on possible COVID-19 exposure?	99.2	0	0.8
Is it important to take patients temperature at the triage before undergoing treatment?	98.5	1.5	0
Should patients be treated on appointment basis only?	87.9	7.6	4.5
Do you think it is important to wear double mask when treating patients?	70.5	15.9	13.6
Is it important for patients to have a preprocedural hand washing before treatment?	94.7	1.5	3.8
Is it important to have a pre-procedural mouth rinse before undergoing any treatment	95.5	1.5	3
Is it important for all patients to have a COVID-19 test before undergoing anything treatment	50	17.4	32.6
Is sanitizer alone sufficient without hand washing to rid the virus?	34.1	55.3	10.6

Do you feel at risk of contacting the infection while working on patients?	92.4	5.3	2.3
Are you stressed to treat patients in clinics?	48.5	8.3	43.2
Are you willing to work in dental clinic during the pandemic?	16.7	53	30.3

Table 2 shows attitude of the students. Majority of our participants (99.2%) agreed on the importance of wearing double masks when treating patients , 98.5% agreed on the importance of checking patient's temperature and 87.9% felt it was important to make an appointment and brief patients before seeing the patients. About 70% agreed on the importance of double masking . About 99% also knew the importance of getting the patients to wash their hands before. Most of our participants (95.5%) believed that preprocedural rinses would help to reduce spread of the COVID-19 virus . However, only 50% of our participants felt that it was necessary for patients to undergo COVID-19 testing before being treated . About 55.3% felt that sanitizer alone was not effective . About 92.4% also felt that that they were at risk of containing the virus when working in clinics.

C. Practices

Table 3: Practices pertaining to infection control during the COVID-19 pandemic

Questions	**Yes(%)**	**No(%)**	**Maybe(%)**
Would you brief patients on the phone prior to treatment?	78.8	11.4	9.8
Would you instruct patients on proper infection control in the dental clinic?	92.4	2.3	5.3
Are you willing to wear complete personal protective equipment before treating any patients?	98.5	1.5	0
Will you wash and sanitize your hands before and after any dental procedure?	98.5	1.5	0
Will you maintain strict social distancing in clinics?	85.6	14.4	0
Would you use high vacuum suction for aerosol generating procedures?	97	3	0
Will you donn and doff in the designated areas ?	99.2	0.8	0
Do you know the orderly sequence to donn before undergoing any dental treatment?	60.6	39.4	0

Do you know the orderly sequence to doff before undergoing any dental treatment?	24.2	75.8	0

Table 3 shows the practices. More than 70% of our participants would brief their patients before appointment with 92.4% giving instructions to their patients on infection controls in clinics . More than 95% agreed to wear strict personal protective equipment when treating patients, practice strict hand washing practices and sanitizing before and after every procedure, use high vacuum suction when carrying out aerosol generating procedures, don and doff in the designated areas before and after dental procedures. However only 60% knew the correct order of donning and doffing.

Discussion:

This study looked into the knowledge of Malaysian dental students about COVID-19 and their attitudes and practices toward treating patients during the pandemic. Dental students in their clinical years are at an increased risk of cross-infection due to the increase of patient contact during their education. Therefore, their knowledge about and attitudes toward standard and extra precautionary measures against COVID-19 are of great importance .Students' knowledge of COVID-19, its symptoms, transmission pathways, transmission control and prevention measures was assessed in this study to identifying the gaps, thus, decreasing the risk and to prepare them for any similar future epidemics. Questionnaire-based studies have been shown to be highly effective in gathering information about dental students' awareness and knowledge of COVID-19, however, careful data collection and interpretation is required.[15] Hence we designed and validated our survey instrument before its use.

Our study results showed that almost all our participants had adequate knowledge towards the nature and transmission of COVID-19 infection in dental clinic. A similar finding was observed in studies conducted among dental students in different parts of the world.[16-19] However, average percentage of knowledge was only about 59.7% in a study conducted in Iran.[20] All our students were aware of the main symptoms of COVID-19, which aids the students in recognizing the threat and taking the necessary precautions during their future dental practice, which is considered a critical part of the virus's management and control. [21] Futhermore, High-speed handpieces have the potential to spread bacteria and viruses to the dentist and dental staff. In this study, 97% of participants were aware of the role of aerosols in viral infection

spread and 97 % said they would use HVE for aerosol generation which further shows their knowledge and carefulness

Similar to other studies [23-25], ours revealed that the majority of the participants were aware of the COVID-19 infection control policies. In a study by Kashif et al, who reported insufficient awareness [26], most of the knowledge gap was associated with donning and doffing PPE procedures. This aspect of his study results are similar to ours, in which only about 60% of our students knew how to donn and doff correctly.

Overall, our students had a positive attitude towards adopting various infection control measures during the pandemic. They also exhibited good infection control practices .The results re similar to some studies conducted. [17,18,19] In this study, clinical dental students' attitude towards wearing PPE including mask, gloves, gown, head cap etc was positive. This could be associated with their fear of contacting infection in clinics and hence to limit the spread of infection . These findings agree with those of a previous study, in which most dental residents feared being infected by contact with patients and preferred to follow the standard treatment guidelines about wearing PPE in their practice. [27]

To summarise, dental students in this study are well-informed about the current pandemic and its potential consequences. While returning to practical learning, they feel an ethical obligation to provide safe treatment to their patients and at the same time ensuring their own safety. The only way to ensure a safe environment for both patients and students is to follow the new strict infection control protocols and to have students re-orientated to these protocols on a regular basis.

Conclusions

Our participants had a good understanding of COVID-19 and the precautions that must be taken to provide adequate dental treatment to patients during the pandemic; however, infection control should be emphasized for not just the clinical students but also preclinical students to ensure knowledge and awareness of this pandemic in ensured and never forgotten.

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Original Research

A Preliminary Institutional Study on Short Term Effects of Dietary Intake and Physical Status in Edentulous Patients after Prosthodontic Rehabilitation.

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ABSTRACT

Background: Edentulism in the elderly population has been related to changes in food intake and nutritional deficiency as it has a measurable impact on mastication. Providing denture helps in enhancing the chewing ability and dietary intake of the elderly population, thus improving their physical status. **Aim:** The objectives for this study were to identify the preliminary changes in physical status of edentulous patients, to identify the changes in calorie intake, and to examine the association between calorie intake with physical status of edentulous individuals before and after prosthodontic rehabilitation. **Methodology**: This cross-sectional study involved 39 patients. The parameters used to conduct the study for evaluating and comparing the dietary intake with physical status were clinical extra oral and intra oral examination of the patients, anthropometric records and 24-hour dietary recall method. **Results**: In 51.3% of the patients, there was increase in calorie intake after prosthodontic rehabilitation. Meanwhile, 28.8% had decrease in calorie intake and 20.5% showed no difference in their calorie intake. For the association between calorie intake with physical status, 33.3% showed an increase in calorie intake and normal BMI. While 25.6% showed a decrease in calorie intake with normal BMI after rehabilitation, 15.4% had no changes in their calorie intake with normal BMI. **Conclusion**: Majority of edentulous patients before and after prosthodontic rehabilitation displayed low calorie intake than the recommended value. There was an increase in calorie intake after rehabilitation while there was no significant difference between calorie intake and physical status of patients before and after prosthodontic rehabilitation.

Keywords: Edentulous, Prosthodontic rehabilitation, Calorie, Nutrition, Physical status

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INTRODUCTION

According to the data from world population prospects: The 2019 Revision, by 2050, one in six people in the world will be over the age 65(16%), up from one in 11 in 2019 (19%). [1] Whilst the Ageing population may stand for the progress in public health efforts, it is still accompanied by inevitable consequences of malnutrition and change in physical status of individuals as an aftermath of edentulism. This is owing to the change in dietary habits, poor dentition and quality and quantity of food consumed. [2, 3]

According to a study the increase in percentage of edentulism is directly related to the advancing age with a marked increase from age of 45 years. [4] The condition of the teeth has a direct relation with changes in food intake and masticatory efficiency. Masticatory ability in turn is affected by presence or absence of teeth, the number of teeth which are functional and use of prosthesis. Edentulism in elderly people has been related to changes in food intake and nutritional deficiency as it has a measurable impact on mastication. Masticatory efficiency is affected by the presence of teeth, the number of functional teeth, and the use of prostheses, which influence the choice of food. [5] They will compensate for the lost teeth and alter dietary intake of the patients positively. [6]

Some studies have shown that providing dentures had significant impact on the oral health quality of life. Thus, it helps in improving the chewing ability and and alter the dietary routine in elderly population. [7, 8, 9] The choice of food quality and quantity are influenced by physical factors such as edentulism, whether total or partial and the use of poorly adapted prostheses. This shows the importance of having a well fitted denture for masticatory purpose. [10] Intake of fruits, vegetables, fibres and protein substantially decreases in individuals who are edentulous in comparison with fully dentate individuals thereby affecting their physical status significantly. [11] Whether or not prosthodontic rehabilitation causes improvement in dietary intake and physical status among edentulous patients in a short time is ambiguous. As the

dietary intakes significantly correlate with physical status. Hence, we aim to investigate the changes in diet and physical status in edentulous individuals before and shortly after provision of prosthesis. A multidimensional parameter has been used to assess the nutritional and physical status including general information of patients, anthropometric records, clinical examination, and 24-hour dietary recall method.

Materials and Methodology

Our research framework included patients attending Lincoln University College Dental Care for prosthodontic rehabilitation. This study aims to evaluate and compare the dietary intake with physical status of edentulous patients before and after prosthodontic rehabilitation.

A cross sectional design was used in this study. The study proposed received its approval by the Institutional Research Ethics Committee, Faculty of Dentistry, Lincoln University College

Target population for our study was patients attending the Lincoln University College Dental Care for prosthodontic rehabilitation.

Inclusion criteria:

- 35 to 75 years old, male and female.
- Partially edentulous and completely edentulous group.
- First time denture wearers and patients who had worn denture before.

Exclusion criteria

- Patients with any physical disabilities that will affect oral functions such as facial palsy, parkinsonism.
- Patients with eating disorders.
- Patients with remaining teeth that are periodontally compromised

The study was done using the following parameters which were evaluated three months after prosthodontic rehabilitation.

Survey Instrument

The parameters included are:

- General information of the patients
- Anthropometric records
- Clinical examination (Intra oral and Extra oral)
- 24-hour dietary recall method

1. General information of the patients

General information of the patients consists of name, gender, age, date of visit, occupation, whether partially or completely edentulous and whether first time denture wearers or had worn denture before has been recorded. This information was recorded in the questionnaire.

2. Anthropometric records

To evaluate the physical status of patients, the following parameters was recorded before and after prosthodontic rehabilitation:

- Height of patients in meters
- Weight of patients in kilograms
- Body Mass Index (BMI)

3. Clinical examination

The examination consisted of checking for common physical signs of deficiency due to poor eating pattern caused by the loss of teeth. Both extraoral and intraoral examination has been carried out.

For extra oral examination, the clinical data included general appearance, skin changes, eyes, pale nails and lips. Intraoral examination consists of evaluating the condition of oral mucosa, presence of any lacerations or ulcers and angular cheilitis.

4. 24-hour dietary recall method

24-hour diet diary recall method was used before and after prosthodontic rehabilitation. Patient had to fill what they ate for breakfast, teatime, lunch, dinner, and supper. Based on the diet diary, the calorie intake of patients before and after prosthodontic rehabilitation was calculated and compared using MyFitnessPal application.

Tool of Analysis

Research Variables

Data was grouped according to demographic profile, type of edentulousness and period of edentulousness.

First Level of Analysis: Descriptive Statistics

All data obtained were entered and analyzed with the aid of computer using Microsoft Excel software and SPSS version 25 statistical software. An extrapolation of data has been

done for the calorie intake and physical status of edentulous individuals before and after prosthodontic rehabilitation. The mean, minimum and maximum value for calorie intake, weight and BMI was described using descriptive statistics.

Second Level of Analysis: Pearson's Chi Square Test

The test of association was done in order to examine the association between calorie intake and physical status of edentulous individuals before and after prosthodontic rehabilitation. The variables accounted were calorie intake pattern (increase, decrease, no change) and BMI (underweight, normal, overweight, obese)

RESULTS

Demographic Profile of Patients

A total number of 39 patients participated in this study. Table 1 shows demographic variables recorded from the patients. Majority of patients were aged 66 to 75 years old (43.59%), followed by age group of 56 to 65 years old (25.64%), 35 to 45 years old (17.95%) and the least patients come from age group of 46 to 55 years old (12.82%). Out of total 39 patients, 53.85% are female while 46.15% are male.

Table 1: Demographic Profile of Patients (n = 39)

Demographic variables		n=39	%
Age groups	35-45	7	17.95
	46-55	5	12.82
	56-65	10	25.64
	66-75	17	43.59
Gender	Male	18	46.15
	Female	21	53.85
Period of edentulousness	< 5 years	12	30.77
	5-10 years	6	15.38
	> 10 years	21	53.84
Demographic variables		n=39	%
Types of edentulousness	Partial	16	41.03
	Complete	10	25.64
	Combination	13	33.33
Had denture before	Yes	25	64.10
	No	14	35.90
Physical disabilities	Yes	0	0
	No	39	100
Eating disorder	Yes	0	0
	No	39	100

Patients participated in this study were classified into 3 groups based on the period of edentulousness, in which 30.77% belonged to group of edentulousness less than 5 years, 15.38% in group of 5 to 10 years and 53.85% of them has been edentulous for more than 10 years. Patients were categorized as partial, complete or combination type of edentulousness which makes up 41.03%, 25.64% and 33.33% respectively. None of the patients had physical disabilities and eating disorders.

Descriptive Statistic of Variables

The Calorie Intake and Physical Status of Edentulous Individuals Before and After Prosthodontic Rehabilitation

The highest calorie intake obtained from 39 patients before they started with prosthodontic rehabilitation was 1828 kcal while lowest calorie intake has shown significant difference which was 570 kcal. The mean weight for patients before starting prosthodontic rehabilitation are 59.84 kg. Since their height also recorded in the study, their BMI which represent their physical status in this study was calculated using formula $\frac{\text{weight (kg)}}{\text{height (m)}^2}$. The highest BMI before denture treatment was 35.38 and 16.33 represent the lowest BMI from those patients.

Based on Table 2, the data collected after prosthodontic rehabilitation showed slight increase in the mean and maximum value of calorie intake, which 1143 and 2000 kCal respectively. On the other hand, minimum calorie intake for patients that had underwent denture treatment showed slight decrease from 570 to 550 kcal. Changes in weight of these patients can be seen from the mean value that had increased to 60.53 kg while the maximum weight has decreased to 86.20 which is not a significant change. For BMI, only minimum value has changes which increased from 16.33 to 16.98.

Table 2: Calorie intake and physical status before and after prosthodontics rehabilitation

	Calorie Intake(kcal)		Weight (kg)		BMI	
	Before	After	Before	After	Before	After
Mean	1131	1143	59.84	60.53	22.82	22.82
Minimum	570	550	48.00	48.00	16.33	16.98
Maximum	1828	2000	86.90	86.20	35.38	35.38

Based on Table 3, after wearing denture, 51.3% of the patients showed increased in their calorie intake. However, there is 28.8% patients who showed decrease and 20.5% had no changes at all in their calorie intake. There is 48.8% of patients who had shown an increase in both the weight and BMI aspect. Meanwhile, 25.6% of them has shown decrease and the remaining 25.6% has no change at all.

Table 3: Changes in weight, BMI and calorie intake before and after prosthodontic rehabilitation (n=39)

	Calorie Intake (kcal)		Weight (kg)		BMI	
	n	%	n	%	n	%
Increase	20	51.3	19	48.8	19	48.8
Decrease	11	28.8	10	25.6	10	25.6
No change	8	20.5	10	25.6	10	25.6

Based on Table 4, there was significant number of 33.3% patient with increase in calorie intake who showed normal BMI after denture treatment. In addition, 25.6% of them that had decrease in calorie intake but normal BMI after prosthodontic rehabilitation, while 15.4% remain in this category despite no changes in their calorie intake. For patients that has overweight body mass index, 12.8% of them had increase in calorie intake. Meanwhile, 2.6% of patient had increase but no change of calorie intake. Although we obtained total of 51.3% of patients that showed increased in calorie intake with various BMI reading, there was no significant association between calorie intake and body mass index (BMI) with p-value = 0.406.

Table 4: Association between calorie intake and (BMI) of edentulous patients after prosthodontic rehabilitation (n=39)

Calorie Intake	BMI								p value
	Underweight		Normal		Overweight		Obese		
	n	%	n	%	n	%	n	%	
Increase	1	2.6	13	33.3	5	12.8	1	2.6	0.406
Decrease	0	0	10	25.6	1	2.6	0	0	
No change	1	2.6	6	15.4	0	0	1	2.6	

DISCUSSION

Our first objective in this study was to identify the calorie intake of edentulous individuals before and after prosthodontic rehabilitation. The mean for calorie intake that was obtained by 24-hour dietary recall method was 1131 kcal. This was before denture delivery. Three-month post denture delivery, the mean for calorie intake was 1143 kcal. The minimum and maximum values of calorie intake before prosthodontic rehabilitation were 570 kcal and 1828 kcal respectively. After denture was issued, the minimum and maximum calorie intake were 550 kcal and 2000 kcal respectively. The result showed that the amount of calorie intake of edentulous individuals before and after prosthodontic rehabilitation in this study were still lower than the recommended calorie intake daily by Ministry of Health Malaysia. For sedentary women and older adults, the recommended calorie intake is 1500 kcal (Malaysian Dietary Guideline). The result was comparable to the study conducted by Pierre et al (2011) which showed that edentulous individuals had calorie intake lower than the recommended dietary intake. [11] Besides, our result also supports the study of Elizabeth et al (1998) which found that calorie-adjusted nutrient intakes decreased with progressively impaired dentition status. [10]

Physical status of edentulous individuals was measured by calculating their Body Mass Index (BMI). The data was acquired from the height and weight of patients that were recorded before and after three months of denture delivery. According to WHO, BMI can be categorized into six categories, as seen in table 5:

The mean weight of patients in this study before starting prosthodontic rehabilitation was 59.84 kg. Average BMI before prosthodontic rehabilitation was 22.82 which indicates normal weight. Majority of the patients also had normal BMI before and after denture delivery. The result was supported by previous study conducted by Katia et al (2008). The authors of the study found out that body composition indicators was generally within normal range, and no statistically significant difference ($p>0.05$) was found between individuals with conventional dentures and implant supported overdenture. [6]

Our second objective of this research was to identify the changes in physical status among edentulous patients before and after prosthodontic rehabilitation. We compared the calorie intake and BMI of edentulous individuals before and after one month of denture delivery to note whether there was any increase or decrease. Post three months of wearing denture, 51.3% of patients showed increase in their calorie intake. The result was in favour with the previous study conducted by McKenna et al (2012). [12] They carried out a longitudinal study that examined subjects before and after denture therapy and reported that MNA scores improved

after denture therapy in subjects with partial dentures. Subjects with complete edentulism also showed improved MNA scores after denture therapy (Prakash et al, 2012). Besides, Prakash et al also concluded that prosthetic rehabilitation of edentulous patients with complete dentures, along with dietary counselling had improved the nutritional status of these patients. [13]

Patients were able to chew better when wearing dentures and this led to increase in daily nutritional intake. Good quality denture group had significantly better masticatory performance than the medium and poor-quality denture groups as discussed by Rosemary et al (2002). [14] For physical status of edentulous individuals, 48.8% of patients had an increase in both the weight and BMI. Meanwhile, 25.6% of them had decrease and the remaining 25.6% had no change in their weight and BMI. There were no significant changes on the body weight and BMI of patients in our study. This was in contrast with previous research conducted by Kanehisa et al (2009). [15] They found out that six months after prosthodontic treatment, body weight changes were significantly different in subjects regardless of denture type used. The situation occured most probably because of the short period of time that we had to assess the weight and BMI of patients which was three months after denture delivery while the previous study conducted assessed the weight changes after 6 months of prosthodontic rehabilitation.

Our result was also not aligned with study carried out by Paturu et al (2011) which found out that there was a significant change in body weight of individuals with complete edentulism after two months of denture delivery. [16] The differences in the result obtained was possibly because the study conducted had emphasized that every complete denture wearer needs to be periodically counseled by a registered dietician and dentist for checkup to avoid malnutrition and disease. Meanwhile in our study, there was no specific professional counselling on tailored nutrition intake for the patients. Thus, proper dietary intake guidance and counselling are of paramount importance for edentulous individuals. In the absence of tailored dietary advice, apparently successful prosthetic rehabilitation does not necessarily result in a satisfactory diet Allen et al (2002). [17] In our third objective, we wanted to examine the association between calorie intake and physical status of edentulous individuals before and after prosthodontic rehabilitation. A percentage of 33.3% of patients had increase in calorie intake and normal BMI after prosthodontic reabilitation. However, 25.6% had decrease in calorie intake and 15.4% had no changes in their calorie intake. Both of these groups also had normal BMI.

Based on these findings, we found out that there was no association between calorie intake and physical status of edentulous individuals before and after three-months denture delivery.

A study had been conducted by Muller et al (2008) which compared the BMI of patients that used conventional denture and implant supported denture after one year. They recorded that all of the subjects have normal BMI and there was no significant difference of BMI after one year of denture delivery. [5] Inconclusive evidence was also recorded in studies investigating the association between dental status and body mass by Kassioni, (2018). Foods such as stringy meats, vegetables and fruits such as carrots and apples are hard to chew, but they are high in nutrients needed by the elderly. [18] High in fat and low in fiber diets by edentulous individuals could be due to reduced mastication ability in those wearing complete dentures Allen et al (2005). This may lead to involuntary weight gain. [19]

In contrast, a prospective study carried out using data from the Survey on Health, Well-being and Aging in Brazil (SABE), had shown that the risk of weight and waist circumference loss was higher among edentulous community of older adults than among dentate ones (Andrade et al, 2014). [20] The National Diet and Nutrition Survey (NDNS) in independent elderly aged 65 years and older in the United Kingdom revealed that having functioning natural dentition of more than 20 teeth increased the possibility of having a normal BMI, while having few natural teeth or being edentulous was associated with a greater risk of being underweight or being obese (Sheiham et al 2002). These discrepancies may be due to cultural or methodological variation in each study. [21] Further, study with a longer period of time and a more sensitive methodology is needed to get more accurate results. Patients should be reviewed after 6 months and 1-year post denture delivery to get proper assessment of the dietary intake and physical status. Moreover, dietary progress should be discussed during each review visit. Nutrition care should be an integral part of the overall prosthodontic treatment as discussed by Rathee and Hooda (2009). [22] Other than that, this study has identified that in most patients there was no significant changes in BMI post denture delivery. This situation would have occurred presumably because the patients were evaluated for changes in physical status after three months period only. The changes in physical status in some patients take time to manifest.

The findings showed that mean value of calorie intake before and after prosthodontic rehabilitation of the denture wearer were less than the recommended value by WHO, it is important for the dentists to educate patients about healthy diet before and after the delivery of denture. It is crucial for clinical dental students to get exposure and obtain knowledge in educating patients about their daily calorie intake as dentists play an important role in detecting and preventing malnutrition in the elderly population. This is especially important in this study as majority of the patients in this study were aged between 66 to 75 years old.

The data acquired from this study has policy implications and can be used by the faculty to identify patients that need dietary counselling. Since most of the patients were not taking enough calorie in a day, they all are indicated for dietary counselling session. With this a holistic treatment approach can be achieved which can benefit the patients significantly

Besides that, the faculty could incorporate the need to take note of patient's anthropometric records such as height and weight so that a general idea of the patient's physical health can be gained and measures can be taken to incorporate a comprehensive customized treatment plan with a focus on patient's diet.

As we only managed to get 39 subjects in this study which is a small sample size, we plan to continue the investigation with a greater sample size. A bigger sample size would give a higher significance and accurate results. Other than that, this study is recommended to be extended to all the dental faculties of thirteen (13) universities in Malaysia so that the results can represent Malaysian population. We also propose a refinement in the methodology to get a more accurate data on nutritional status and physical status of patients before and after prosthodontic rehabilitation. Few methods can be used such as Mini Nutritional Assessment (MNA), and Geriatric Oral Health Assessment Index (GOHAI).

CONCLUSION

This study helped in identifying the nutritional status among edentulous individuals that came to LUC Dental Clinic for prosthodontic rehabilitation. Their overall physical status was obtained through Body Mass Index (BMI) and clinical examination. This gives us the general health status of edentulous patients that came to seek treatment. It also helps us to assess approximately the amount of calorie intake taken by them. Since most of the edentulous patients are the elderly, they are at a higher risk of malnutrition as mentioned by Mafauzy (2000). [23] Thus, this study enables us to identify patients who are at risk of malnutrition.

There are not many studies conducted to find out the association between nutritional intake and physical status of denture wearers in Malaysia. We hope that this study will be continued and can be improved by others in the future to get more significant and accurate results.

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Original Research

Microbial Growth Inhibitory Activities of Skin and Seed Extracts of *Pisum sativum*

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Abstract

Introduction: The improper use of antibiotics to treat various bacterial infections occurring in humans had caused the rise of antibiotic-resistant bacterial strains. These lead to an urgent need in developing novel antimicrobial compounds. **Objective:** The extracts of the skin and seeds of *Pisum sativum* were screened for antimicrobial activity against two different species of Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and one species of Gram-negative bacteria (*Escherichia coli*). **Materials and methods:** Methanolic crude extracts were prepared at different concentrations. Their antimicrobial activities were screened by disc diffusion assays and minimum inhibitory concentration (MIC) determination assays. **Results:** Antimicrobial inhibitory effects were observed in *B. subtilis*. However, further investigation is needed to identify the reasons of *S. aureus* and *E. coli* being resistant towards both types of phytochemical extracts. **Conclusions:** Our findings support the hypothesis that the skin and seeds of *P. sativum* possess antimicrobial properties. Subsequent studies should be driven towards the identification of key phytocompounds, which can be potentially developed into new plant-based antimicrobial agents.

Keywords: Antimicrobial activities; Methanolic extracts; *Pisum sativum*

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Introduction

Antibiotics are medications that are widely used in the management of pathogenic infections. The increasing concerns on antibiotic-resistant bacterial strains have gathered research focus on phytochemicals which carry antimicrobial activity. As a valuable source of

bioactive compounds with potent antimicrobial activities, phytochemicals possess different chemical classes which could reverse the antibiotic resistance [1].

Pisum sativum (also known as garden pea or green pea) is a species originates from *Fabaceae* family, which usually consists of a pod where it contains several small spherical seeds. One of the first antimicrobial activities of *P. sativum* reported was the phenolic extracts of sprouted peas against *Helicobacter pylori*, a Gram-negative microorganism that leads to gastric ulcers [2]. The same study also reported that *P. sativum* exhibited a better antimicrobial property when its cotyledons were treated with aspirin.

Limited studies were conducted to investigate antimicrobial activities of *P. sativum* against bacteria. In 2014, the peel extracts of *P. sativum* exhibited antimicrobial activities against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonela enterica* [3]. In this study, the researchers showed that the methanolic extracts of *P. sativum* carried antimicrobial activities, while the aqueous extract did not. The findings were in accordance with some earlier studies reporting antimicrobial activities of *P. sativum* extracts derived from seeds and skins [4]. In another experiment, Nair *et al.* reported that the phytolectins isolated from the seeds of *P. sativum* exhibited antimicrobial activity against several bacterial species, including *P. aeruginosa* [5]. In recent years, the extracts of *P. sativum* have been used in green biosynthesis of silver nanoparticles in an effort to explore its antidiabetic, cytotoxicity, antioxidant, and antimicrobial activities against foodborne pathogens[6,7]. Its potential applications in nutraceutical formulations and food packaging have also been explored in some recent research articles [8,9]. Nevertheless, fundamental questions such as types of extraction and their specific antimicrobial abilities remained elusive.

Due to the limited information available on the antimicrobial properties of *P. sativum*, the methanolic extracts of its skin and seeds were of our interest in this study. The findings fill the existing research gaps in identifying novel modern antibiotics to treat microbial infections, especially those which caused by bacteria. Hence, this study aims to determine the level of antimicrobial activities in the extracts of skin and seeds of *P. sativum*.

Materials and methods

Procurement and preparation of samples

P. sativum was precured from a local grocery store located in Kota Damansara, Malaysia. The samples were opened using a sterile scalpel, and the seeds were removed from

the pods. The skin and seeds were placed separately in two sterile beakers and allowed to dry in an oven (50 ℃, 72 hours).

Preparation of microorganisms

Three types of microorganisms were used in this study. They were *Staphylococcus aureus* (cocci, Gram positive), *Bacillus subtilis* (rod-shaped, Gram positive) and *Escherichia coli* (rod-shaped, Gram negative). These microorganisms were cultured and maintained on Nutrient agars under aseptic environment.

Extraction of skin and seeds of *P. sativum*

The skin and seeds of *P. sativum* were dried (50 ℃, 72 hours) and ground into smooth powder form using a mortar and pestle. The weights of these powdered samples were recorded and placed in a cleaned conical flask. Methanol (100 mL) was added to cover the samples completely. Then, the conical flasks were sealed with parafilm, immersed partially into a beaker pre-filled with paraffin oil, which was placed on a hot plate set at 45 ℃. Using magnetic stirrers, the mixture was constantly mixed and heated at 45 ℃ for 72 hours.

Filtration of extracts

After extraction, the mixture was filtered using a filter funnel and a Whatman filter paper. The filtrate obtained was placed in a cleaned beaker, and incubated at 45 ℃ until it was completely dried. The weight of the dried extract was calculated by subtracting the total weight (beaker with the dried extract) with the original weight of empty beaker.

Reconstitution of extracts

The dried extracts were reconstituted with 2 mL of methanol. Mild sonication was applied to help dissolving the dried extracts. After the extracts have dissolved completely, the concentrations of methanolic extract were calculated and stored in -20 ℃ until used.

This procedure was repeated for determination of minimum inhibitory concentration (MIC), with the 2 mL of methanol substituted by 2 mL of sterile water.

Antimicrobial disc diffusion assays

Microbial broth cultures of all three microorganisms were prepared to 0.5 McFarland standard. Under aseptic conditions, 100 µL of each microorganism was transferred and spread

evenly onto Nutrient agars. All inoculated Nutrient agars were let dried before discs with extracts were placed.

Discs were prepared from Whatman filter paper using a paper puncher. The discs were autoclaved and dried before used. 10 µL of extracts were transferred onto each disc and allowed to dry in a biosafety cabinet. Dried discs were visually inspected before placed onto the surface of inoculated Nutrient agars. After that, all inoculated Nutrient agars with discs were incubated at 37 ℃ for 16 hours. Then, the diameters of inhibition zones were measured (in mm). All samples in this procedure were prepared in triplicates.

Determination of minimum inhibitory concentration (MIC)

MIC was conducted on *B. subtilis* as it showed promising results in antimicrobial disc diffusion assay.

Two sets of *P. sativum* skin and seed extracts with a series of different concentrations were prepared. Each sample was diluted by half using sterile Nutrient broth. After which, 10 µL of *B. subtilis* (prepared to 0.5 McFarland standard) were inoculated into each tube. These tubes were then incubated at 37 ℃ for 16 hours. Microbial growths were observed visually and on fresh Nutrient agar plates after that.

Statistical analyses

All statistical data were compiled and recorded in Microsoft Excel. Standard deviations and One-way ANOVA (reported in *P*-values) were conducted using the same software based on data obtained from samples prepared in biological triplicates.

Ethical clearance

Ethical clearance for this research project was granted by SEGi University Research Ethics Committee, with reference number SEGiEC/SR/FOP/12/2020-2021.

Results

Skin extract of *P. sativum*

The skin extracts of *P. sativum* were prepared in four independent assays. These extracts were reconstituted in methanol. The concentrations obtained were 0.12 g/mL, 0.28 g/mL, 1.03 g/mL, and 1.85 g/mL.

Seed extract of *P. sativum*

The seed extracts of *P. sativum* were prepared in three independent assays. These extracts were reconstituted in methanol, with concentrations recorded at 0.17 g/mL, 0.37 g/mL, and 0.51 g/mL.

Antimicrobial disc diffusion assays

For *P. sativum* skin extracts, our results indicated that only *B. subtilis* was susceptible to these samples, while *E. coli* and *S. aureus* had no visible inhibition zone was observed. Overall, the size of inhibition zones was in proportion to the concentrations of the extracts used in disc diffusion assays (Table 1). In addition, the concentration of skin extract was found to be significant in altering the antimicrobial activity of our samples ($P < 0.05$).

Table 1: Average diameters of inhibition zone (mm) of four *P. sativum* skin extracts against the test microorganisms.

Bacteria	Average diameters of inhibition zone (mm) / Concentrations of skin extracts (g/mL)			
	0.122 (g/mL)	0.277 (g/mL)	1.03 (g/mL)	1.85 (g/mL)
E. coli	0	0	0	0
S. aureus	0	0	0	0
B. subtilis	7 ± 0	10.7 ± 1.15	9.7 ± 1.5	10.7 ± 0.58

For *P. sativum* seed extracts, no visible inhibition zone was observed for *E. coli* and *S. aureus*. In addition, the concentration of extract at 0.16 g/mL did not show any antimicrobial inhibitory effect against *B. subtilis* (Table 2). However, as the extract concentrations increased to 0.37 g/mL and 0.51 g/mL, the growth of *B. subtilis* was inhibited (Table 2).

Table 2: Average diameters of inhibition zone (mm) of three *P. sativum* seed extracts against the test microorganisms.

Bacteria	Average diameters of zone of inhibition (mm) / Concentrations of seed extracts (g/mL)		
	0.16 (g/mL)	0.37 (g/mL)	0.51 (g/mL)
E. coli	0	0	0
S. aureus	0	0	0

B. subtilis	0	10.7 ± 0.58	12.3 ± 0.58

Determination of minimum inhibitory concentration (MIC)

Table 3 indicates the possible range of MIC where *P. sativum* skin or seed extract may be able to inhibit the growth of *B. subtilis*. For *P. sativum* skin extract, the range of concentration fell between 0.06 - 0.12 g/mL, whereas for the seed extract, it fell between 0.03 – 0.06 g/mL.

Table 3: Microbial inhibitory concentration (MIC) determination for *P. sativum* skin and seed extracts.

***P. sativum* skin extract**		***P. sativum* seed extract**	
Concentration (g/mL)	Presence of *B. subtilis* colonies on Nutrient agar	Concentration (g/mL)	Presence of *B. subtilis* colonies on Nutrient agar
1.85	No	0.51	No
0.93	No	0.26	No
0.46	No	0.13	No
0.23	No	0.06	No
0.12	No	0.03	**Yes**
0.06	**Yes**	0.02	**Yes**
0.00	**Yes**	0.00	**Yes**

Discussion

The methanolic skin extracts of *P. sativum* exhibited its antimicrobial effect against *B. subtilis*, while the same effect was not detected on *S. aureus* and *E. coli*. These findings were consistent with several previous studies although the strengths of inhibitions varied [3,4]. In our opition, such observations drew a clear distinction between spore-forming (*B. subtilis*) and non-spore forming (*S. aureus* and *E. coli*) bacterial species. Although additional purification processes and tests are required for our *P. sativum* methanolic skin extracts, at this stage, it is our speculation that the product extracted contained sporicidal compounds which have been previously reported by other researchers [10,11]. In addition, our results indicated that the concentration of *P. sativum* skin extract carries a statistically significant role in exhibiting its antimicrobial properties ($P < 0.05$). These findings highlighted the needs in identifying the key

phytochemical compounds in the skin of *P. sativum* that inhibit the growth of both Gram-positive and Gram-negative bacterial cells. In addition, it will be of researchers' interest in understanding the biomolecular interactions between these compounds and *B. subtilis*, which was once known as the causing agent of several serious infections [6], with a recent detailed study on its virulence potential which may be lethal to vertebrate animals [7].

Although the methanolic seed extract of *P. sativum* exhibited a similar trend towards the three tested microorganisms – *E. coli*, *S. aureus*, and *B. subtilis*, the strength of microbial inhibition of seed extract was reported higher than that of skin extract. This conclusion was made based on the minimum inhibitory concentration (MIC) assays, where the possible range of MIC recorded for skin and seed extracts were 0.06 – 0.12 g/mL and 0.03 – 0.06 g/mL, respectively.

It is worth highlighting that a previous study by Nair *et al.* has presented the antimicrobial effect of phytolectin extracted from the seeds of *P. sativum*. In the study, researchers extracted and purified pure phytolectin which were later reported to possess inhibitory effect against *E. coli*, *S. aureus* and *B. subtilis* [5].

In our study, we are confident that the phytocompounds that inhibit the growth of *B. subtilis* were found in the methanolic crude extracts of skin and seed of *P. sativum*. This again highlights the needs of purifying individual phytocompounds for subsequent detailed studies. The discovery of biomolecular interactions between these candidates and microscopic pathogens can be of great contributions towards the discovery and development of antimicrobial biopharmaceuticals.

Conclusion

In conclusion, the extracts of the skin and seed of *P. sativum* exhibited their antimicrobial properties against *B. subtilis*. However, based on our findings, the same samples did not exert any detectable antimicrobial activity against *E. coli* and *S. aureus*. This report serves as one of the first research findings focusing on antimicrobial properties of *P. sativum*, with parallel screenings on Gram-positive and Gram-negative bacteria in a single experimental design. Nevertheless, further investigations using various methods of phytocompound extraction should be conducted in near future.

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