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MECHANICAL PROPERTIES AND ANTIFUNGAL ACTIVITY OF GANODERMA LUCIDUM MODIFIED POLYMETHYL METHACRYLATE: AN IN-VITRO STUDY

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ABSTRACT

Background: Polymethylmethacrylate (PMMA) is a common material used in dentistry. However, it has many deficiencies that could be addressed. Ganoderma Lucidum (G. Lucidum) material has a desirable antifungal effect and may also enhance the mechanical strength of PMMA materials.

Objective: This study aimed to assess the flexural strength, modulus of elasticity and antifungal activity of the PMMA modified by G. Lucidum.

Material and methods: Forty-eight specimens were prepared as two groups, group 1: PMMA (control), group 2: PMMA modified by G. Lucidum. Flexural strength and antifungal activity were assessed followed by scanning by confocal laser scanning microscopy (CFLSM). Modulus of elasticity was also calculated for both groups. Data were collected and statistically tested using one-way analysis of variance (ANOVA) to check the difference between studied groups then pairwise comparison by post-hoc test (Bonferroni's) was applied.

Result: Group 2 showed higher flexural strength and modulus of elasticity (66.63155± 8.99886 MPa), (3243.82±732.2 MPa) respectively. Broth micro dilution assay of group 2 showed the microbial inhibited concentration (MIC) values were ≥ 375 µg/ml for Candida Albicans. This was also confirmed by the (CFLSM) by showing increased dead C. Albicans cells.

Conclusion. It can be reported that the modification of PMMA with G. Lucidum can increase some mechanical properties and antifungal activity of the PMMA material.

KEYWORDS: Polymethylmethacrylate, Ganoderma Lucidum, flexural strength, confocal microscopy

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INTRODUCTION

Polymethylmethacrylate (PMMA) is a common and versatile material used mainly for removable prosthodontics and orthodontic appliances [1]. Its widespread application in the dental field is because of its good esthetics, obtainability, low cost, processing and repair simplicity [2]. Although PMMA displays useful characteristics, its deficiencies have created a great demand for development.

One of the biggest challenges that comes with using this material is that it easily gets contaminated with microorganisms from the oral environment that adversely deteriorate patients' health. Several trials have been made to improve the properties of PMMA and give it antimicrobial properties through fresh additions [2, 3].

Candida Albicans (C. Albicans) has been found to colonize and cause infections in the oral cavity, particularly in denture wearers. The causes of denture stomatitis have not been inspected. Effective therapy of denture stomatitis requires not only treating the tissues but also disinfecting the denture. Antimicrobial agents added to the dentures could reduce the intensity and inflammation of stomatitis by inhibiting C. Albicans [4].

Traditional Chinese medicine has historically employed reishi mushrooms to promote health, longevity, and spiritual growth [5]. Reishi mushrooms were named by "Shen Nong of the Shu Dynasty" which was later considered as a "premium herb". He clarified it could be taken regularly without adverse effects and has been used as a powerful immunological stimulant that defends the entire body [6]. Only six of the over 2000 Reishi classes known to date have been studied for potential health-beneficial properties: yellow, red, blue, black, white, and purple type. The most effective and healthy types have been seen in red Reishi (G. Lucidum) and black Reishi (G. Sinensis) [7].

Ganoderma Lucidum (G. Lucidum) encompasses more than 400 bioactive compounds, such as sterols, steroids, polysaccharides, triterpenoids, nucleotides, proteins/peptides and fatty acids, which included in a variety of medical applications [8, 9]. In many countries, Ganoderma Lucidum plays an important role in the treatment and prevention of various diseases [10]. It has a successful medicinal and nutraceutical application. It has a role in the health supplement and cosmetics industries [11]. Many preclinical and clinical trials were conducted to investigate its therapeutic applications. It was recognized as antioxidant, anticancer, anti-inflammatory, carcinostatic, anti-allergic, antiviral, antifungal and antibacterial agents [11-15]. Triterpenoids and polysaccharides are the most prominent pharmacologically active compounds. The mineral content is mainly encompassing magnesium, calcium, selenium, phosphorus, iron, zinc, potassium and copper [16].

Mechanical properties are important criteria for evaluating and determining the longevity and quality of service provided by an appliance, despite the importance of developing a PMMA that is antimicrobial resistant. High mechanical properties protect the appliances from scratches, wear and sudden fractures because of uneven masticatory forces [12,13,17].

There is insufficient evidence for using natural products to treat denture stomatitis, especially by using it as an ingredient of the denture material [18]. Moreover, no studies showed the effect of integrating these natural products into the mechanical properties of the PMMA. Thus, studies that incorporate novel, biocompatible, and beneficial natural products such as G. lucidum are encouraged. Therefore, this study was conducted to test the influence of modifying PMMA composition by the antifungal G. Lucidum mushroom extract on the flexural strength, modulus of elasticity and antifungal property of PMMA. In this study, our hypothesis is that there is a statistically significant increase in the flexural strength, Young's modulus and the antifungal activity of the acrylic resins modified with G. Lucidum extract.

MATERIALS

Polymethylmethacrylate (PMMA) powder and methylmethacrylate (MMA) liquid (Acrostone, Egypt) (chemically cured), was used as a denture base material. Ganoderma Lucidum was prepared by ball milling and added as inorganic fillers in the denture base material.

Ganoderma mixing method:

Natural extract sachet powder, grain size < 0.125 mm, is used (Ganozhi, DXN, Malaysia). Experimental material powders were prepared by mixing 10% (w/w) with the acrylic resin powder of PMMA. This ratio was selected according to (IC50) of Ganoderma against Candida Albicans [19]. The half-maximal inhibitory concentration (IC50) is a measure of the effectiveness of a Ganoderm in inhibiting biological/biochemical function of Candida Albicans. The powder was weighed on a balance (up to 0.001 g accuracy) (PS.R1, RADWAG, England). The mixing process was carried out by using a machine with ball-mill (planetary-ball-mill-pm-400) for 30 min at 250 rpm speed to ensure distribution of the phases homogeneously. The size of the Ganoderma particles didn't change before and after ball milling. Experimental materials were prepared and incorporated in the PMMA powder in a specialized nanotechnology research laboratory for producing and studying nanomaterials (Nano Gate Co., Egypt).

METHODOLOGY

1. Sample size calculation

Sample size was calculated by power analysis software (G*Power v3.1.9.2; Henrich-Hein-University Dussldorf, Dussldorf, Germany) (n = 8; effect size [f] = 3.96; Beta level (β) = 0.95; power = 95%; α = 0.05).

2. Specimen grouping

Forty-eight specimens of acrylic resin were prepared (24 for each group). Specimens were categorized into 2 groups: group 1 (control), the unmodified acrylic resin, and group 2, the G. Lucidum modified acrylic resin. Eight specimens were assigned for each test in each group. The specimens were tested for flexural strength, antifungal activity and Confocal Laser Scanning Microscopy.

3. Mold fabrication

A mold of industrial silicon, with 4 cuboid housings, was fabricated at (65 mm (length) × 10 mm (width) × 2.5 mm (depth) dimensions for each housing. [20]

4. Specimen preparation

The powder and the monomer of each group were mixed in a clean dry mixing jar following the manufacturers' instructions. The mixing process was carried out until the dough stage was reached. Then, packing with sufficient pressure inside the molds was performed until mold overfilling. The acrylic resin was allowed to fully cure before being removed from the mold. The previous steps were repeated to have 48 specimens (24 in each group). All specimens were kept in distilled water for 10 days at room temperature and then retrieved before testing.

Testing

A. Flexural strength test

Eight specimens of group (1) and eight specimens of group (2) were tested. The specimens' flexural strength was assessed using a universal testing machine's by 3-point loading test. (Instron model 3345, England). Using computer software, the data was recorded. (Bluehill Instron, England). The specimens were positioned on supporting jigs 40 mm apart. A centrally-located plunger with a diameter of 20 mm was used to apply a loading force, after which the maximum load was applied to the specimen at a crosshead speed of 5 mm/min until fracture. The flexural strength was calculated

as (F=3PL/2bd2), where F is the flexural strength, L is the support span length, P is the applied load, b is the specimen and d is the sample thickness. (Fig.1)

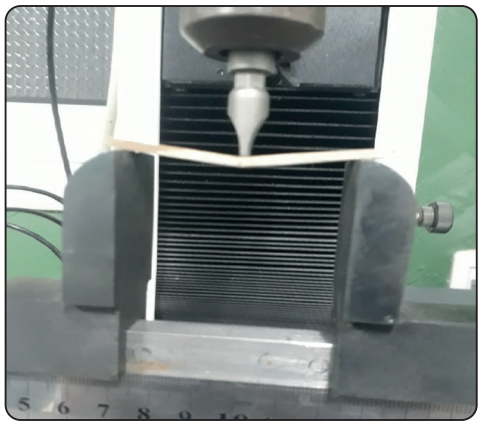

Fig. (1): Universal testing machine holding a specimen for the 3-point loading test.

B. Modulus of elasticity calculation:

The flexural modulus (MPa) was calculated for eight specimens of each group according to the equation: (Ebend=PL3/4Dbd3), where Ebend is the flexural or bending modulus of elasticity equivalent to Young's modulus (E) and D is the deformation.

C. Broth microdilution assay (test)

Antifungal agent preparation

Fluconazole (Sigma-Aldrich- USA) was dissolved in N, N-dimethylformamide (Sigma-Aldrich- USA) to make a 10.0 mg/ml stock solution.

Eight specimens of group (1) and eight specimens of group (2) were tested. A broth microdilution assay using was done using a resazurin dye (Alamar Blue) [21]

The 96-well plates were set by dispensing an extract. This extract contained 95 µL of nutrient broth and 5 µL of the inoculum was added into each well. Each extract was prepared at a concentration of 0.166 (v/v) and added to the first well, followed by a two-fold dilution until the ninth well. The 10th column wells were filled with MH broth (195 µL) and kept for controlling of the fungal growth, whereas the wells in column 11 were reserved for controlling of the broth sterility. The negative control wells were set in the last column and included 5 µL of the inoculum and 195 µL of nutrient broth. The plate was tightly closed and incubated for 24 h at 37 °C in microaerophilic conditions. Plates were visually read after incubation to look for turbidity, which is a sign of microbial growth. The lowest antifungal concentration that inhibited microbial growth was determined as the MIC value.

C. Confocal Laser Scanning Microscopy (CLSM)

Eight specimens of group (1) and eight specimens of group (2) were examined. The Candida Albicans (C. Albicans) biofilms adhered to the PMMA specimens were stained by Film Tracer TM LIVE/ DEAD Biofilm Viability kit (Invitrogen, USA). To distinguish between live and dead fungal cells, two fluorescent dyes were used. Syto 9 dye was used to stain live C. Albicans cells for green fluorescence, and propidium iodide (PI) dye was used to stain dead cells with compromised cell membranes for red fluorescence. The dyes were used according to the manufacturer's instructions in a 1:1 ratio. Each disc received just 500 mL for 20 minutes in a dark room at room temperature, followed by rinsing with sterile distilled water.

The Syto 9 stain excitation/emission wavelength was about 480/500 nm and 490/635 nm for with a source of illumination (PI stain with Kr/Ar). A 63x water immersion lens attached to a confocal laser scanning microscope was used to image randomly selected areas (LSM 710. Software Ver. ZEN 2.3; Carl Zeiss (ZEISS), Jena, Germany).

Statistical analysis

The Statistical Package for Scientific Studies was used to conduct the statistical analysis. (SPSS Ver. 16.0; IBM, Chicago, USA) (α = 0.05). The descriptive statistics were calculated as means and standard deviation followed by testing the normality

by the Shapiro-Wilk test. The data were parametric, so a one-way analysis of variance (ANOVA) test was used to compare studied groups. Bonferroni's post-hoc test for pair-wise comparison then was applied.

RESULTS

Flexural strength

The mean value and standard deviation of the flexural strength in Group 2 (66.63155± 8.99886 MPa) were higher than the value of group 1 (59.23± 6.01560 MPa). There was a statistically significant difference between the two tested groups ($p<0.05$).

Modulus of elasticity

The mean value for modulus of elasticity recorded in samples of group 2 (3243.82±732.2 MPa) was higher than the mean value of group 1 (1705.95±259.72 MPa). There was a statistically significant difference between the two tested groups ($p<0.05$).

Broth microdilution assay

Fluconazole had high anti-C. albicans activity when compounded directly into Ganoderma Lucidum. MIC values for group 2 were ≥ 375 µg/ml for C. albicans ATCC 64124 and HMV4C. The MIC test values are shown in table (1)

TABLE (1): MIC (µg/ml) of G Lucidum determined by visual reading.

conc (µg/ml)	1000	500	250	125	62.5	+ control	- control
Result	-	-	+	+	+	+	-

Confocal Microscopy Investigation

Selected images of the live/dead assay taken with fluorescence confocal microscopy are shown in Figure (1). The live C. albicans showed as green, while the dead cells were seen in red. The non-modified PMMA showed few dead fungi visible in the specimens in heavy, complete, and dense coverage of live fungi (Fig.2 A). For the G.L-modified PMMA specimens, the fraction of the dead fungi (red color) becomes more clear and predominant than the first non-modified group (Fig.2B)

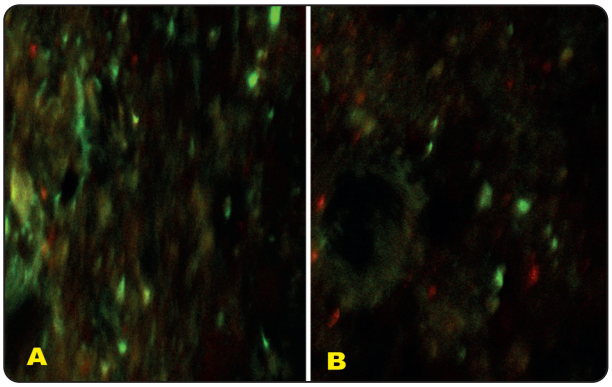

Fig. (2): Images of live/Dead C. Albicans biofilms at 3 days by staining confocal microscopy (X60). Life fungi (green) shown as coverage of dense biofilm with traces of dead fungi (red) scattered on the non-modified PMMA (A). For the G.L modified PMMA specimens (B), the contribution of the (red) dead fungi has been recognizable. The dead fungi became more prevalent, with only a few patchy areas of green.

DISCUSSION

The current study modified the composition of PMMA with a natural extract of G. Lucidum. Specimens were prepared and examined their flexural strength, modulus of elasticity and antifungal activity. The findings revealed that the G. Lucidum modified PMMA material has an influence on the flexural strength, modulus of elasticity and antifungal activity, which means accepting the study hypothesis.

The results showed that the addition of G. Lucidum had a statistically significant improvement in the flexural strength of the acrylic resin and this may be beneficial for denture base reinforcement. This finding will be valuable for patients suffering from frequent denture fractures, especially during mastication. This improvement in mechanical

properties was attributed to the mineral content of G. lucidum, which might act as fillers responsible for reinforcing the PMMA set material [22, 23]. Moreover. G. Lucidum carries many hydroxyl-groups and carboxyl-groups which might bind to their counterpart in the PMMAs by forming hydrogen bonds. This binding is crucial to avoid stress concentrations around the fillers and enforce the PMMA macrostructure leading to higher mechanical performance [24, 25]. These findings are in the same line with the study conducted by Carlo et al. [26] They demonstrated that the addition of graphene to PMMA leads to a significant increase in flexural strength and modulus of elasticity. In the same context, another research showed improvement in mechanical and biological properties by adding G. Lucidum to PMMA [27].

The investigated images of the confocal microscopy of the current study showed that modifying PMMA with G. Lucidum led to a decrease in the C. Albicans biofilm thickness and density. That meant an improvement in the antifungal property when compared with the non-modified PMMA. This might be because of G. Lucidum being a polysaccharide-protein complex. It is non-toxic and biocompatible, as well as a natural antifungal agent [10].

It has another antifungal activity by inhibiting (1-3)-glucan synthesis, which resulted in a disordered and osmotically unstable cell wall. That caused fungal cell death and/or reduced host tissue damage [28,29]. Inhibition of (1-3)-glucan synthase also affected several other essential components of the fungal cell membrane, such as ergosterol content, and the cell wall content including chitin and ultrastructural alterations [30]. These findings agreed with Onishi et al. study, they conducted a study and discovered four triterpenoids, which could inhibit glucan synthase, present in G. Lucidum [30,31]. Accordingly, G. Lucidum has a good potential inclusion in the denture base material to inhibit C. Albicans integrity.

The results of the current study coincided with Gad et al. [32]. They conducted an in vitro study to enhance the antifungal property of the PMMA denture base material. They could successfully incorporate an antifungal extract using a similar methodology as the current study. They claimed that their modified PMMA denture base material had a novel antifungal property.

On the other hand, Walczak et al. [33] reported that, while chitosan salts had antifungal activity, adding chitosan salt powders to denture base materials had no antifungal, antibiofilm, or anti-adherent effects.

In addition, further study by Jing's research contradicted the results of the present study. They found that tea extracts could significantly reduce the growth of C. Albicans in the culture medium, but they had no effect on C. Albicans biofilms when was added to PMMA resin surface [34]. Further studies considering the clinical trials and using other forms of PMMA materials are required. The denture base material used in the study was a chemically cured material to avoid any adverse effect of curing temperature on the studied specimens. Testing different concentrations of the experimented material are also required. Further in vitro and clinical studies should be conducted on heat-cured PMMA. Further important mechanical tests for a denture base material, such as impact strength and hardness, are also encouraged.

CONCLUSION

Based on the results of the research presented, adding the G. Lucidum as an ingredient of the denture base material showed promising mechanical and antifungal characteristics for further use in experimental and clinical investigations.

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