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Estimation the Antibacterial Activity of Triterpenoids and Polysaccharides Extracted from *Ganoderma lucidum* Mushroom

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Abstract: *Ganoderma lucidum* has gained recognition as a significant medicinal mushroom due to its abundance of highly bioactive secondary metabolites that possess a wide range of chemical structures. Polysaccharides and triterpenoids were considered the first bioactive ingredients which were used to control several diseases. In this study, the fruiting bodies powders were used to extract triterpenoids (GLTs) and polysaccharides (GLPs) from this mushroom. HPLC was utilized to detect GLTs and GLPs by using Ganoderic Acid (A) and β-Glucan respectively as standards. To estimate the antibacterial efficacy of these extracts, four bacteria were utilized which demonstrated resistance to antibiotics. From 1000 gm fruiting bodies that were used to extract each of GLTs and GLPs, 16.1 g GLTs and 15.7 g GLPs were extracted. The results of HPLC analysis exhibited that the concentration of β-Glucan in GLPs was 416.25 µg and 985 µg per gram of Ganoderic acid in GLTs. The antibacterial activity of these extracts showed that GLTs and GLPs have high effects against two gram-positive and two gram-negative bacteria and the concentration of 100 mg/ml of GLTs exhibited the most substantial inhibitory zones against bacteria, measuring 16.33 mm, 19.33 mm, 13 mm, and 15.33 mm for *E. faecalis*, *S. aureus*, *E. coli*, and *P. aeruginosa* respectively. On the other hand, 100 mg/ml of GLPs significantly inhibited all examined bacteria, yielding the maximum inhibition zones of 15 mm for *E. faecalis*, 19.66 mm for *S. aureus*, 13.33 mm for *E. coli*, and 14.33 mm for *P. aeruginosa*. These results suggest that both GLPs and GLTs possess promising antibacterial properties against antibiotic-resistant bacteria and demand further research to clarify their mechanisms of action and investigate their potential as novel therapeutic agents.

Keywords: *Ganoderma lucidum*, Polysaccharides, Triterpenoids, Gram-positive, Gram-negative

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Introduction

Ganoderma lucidum (*G. lucidum*) is one of the most important medicinal mushrooms which was discovered over two thousand years ago and was used in traditional medicine for several decades. *G. lucidum* has gained recognition as a significant medicinal mushroom due to its abundance of highly bioactive secondary metabolites that possess a wide range of chemical structures (1). The active compounds obtained from the fruiting bodies, mycelia, and spores consist of phenols, terpenoids, steroids, and nucleotides in addition to glycoproteins and polysaccharides. Polysaccharides and triterpenoids were considered the first bioactive ingredients which were used to control several diseases (2).

Polysaccharides are a chain of sugar molecules joined by glycoside bonds. Several types of polysaccharides were extracted from *G. lucidum* with high molecular weights ranging from thousands to millions Dalton. Glucose sugar molecule is the major component together with other sugar molecules like xylose, mannose, galactose, and fructose. *G. lucidum* polysaccharides (GLPs) can stimulate immunological responses with differing immune benefits (3). GLPs chains can form a triple helix which is stabilized by hydrogen bonds. This triple helix structure of GLPs was exhibited in β-D-glucans. The biological activity of β-D-glucans is determined by these beta structures. The chemical structures of GLPs vary depending on the extraction method (4, 5).

On the other hand, triterpenoids are a type of terpenes which consist of six isoprene units. The isoprene units of triterpenoids may form linear chains or ring structures. More than twenty thousand triterpenoids were identified in herbs such as squalene-, lanostane, dammarane, lupine, oleanane, ursane, and hopane (6). The chemical structures of *G. lucidum* triterpenoids (GLTs) are derived from lanostane, synthesized through the squalene cycle. GLTs are classified into two groups: ganoderma alcohols, due to the presence of the hydroxyl group, or ganoderic acids, due to the presence of the carboxyl group on the side chain of the lanostane moiety (7). Most GLTs consist of 30 or 27 carbon, and a few have 24 carbon atoms (8). The first triterpenoids isolated from *G. lucidum* in 1982 were ganoderic acid A and ganoderic acid B (9).

Over the past decades, there has been a significant rise in the quest for novel therapeutic bioactive chemicals that can function as antimicrobial agents. This is primarily driven by the emergence of antibiotic resistance in microbes that cause diseases in humans, and are responsible for millions of deaths annually. Hence, there is a pressing requirement for novel, cost-effective medications that can exhibit prolonged efficacy before the onset of resistance. The identification of novel compounds that demonstrate significant efficacy against pathogenic microorganisms, including toxigenic staphylococci, anaerobic bacteria, *pseudomonas*, different pathogenic fungi, and others, without developing resistance to current antibiotics (10, 11).

Consequently, triterpenoids, lectins, polysaccharides, and other similar substances of *G. lucidum* mushroom are types of these novel and cost effective compounds and classified as antimicrobial agents. They exert their effects on the bacterial cytoplasmic membrane. The chemicals included in *G. lucidum* can impede the growth of both gram negative and gram positive bacteria (1). Hence, the aims of the study are to extract the triterpenoids and polysaccharides from local fruiting bodies of *G. lucidum* mushroom, detect these extracts by HPLC, and estimate the antibacterial activity of these extracts against four multi-drug resistance bacteria.

Materials and Methods

Mushroom fruiting bodies

This study used Iraqi *G. lucidum* fruiting bodies obtained from the National Center of Organic Farming, Iraqi Ministry of Agriculture, Baghdad, Iraq. The fruiting bodies were dried at 45 °C for two days by using a laboratory drier (Memmert, England). The dried fruiting bodies have been ground into fine powders using a laboratory grinder (Model FW100, Brand Carlssoon Technologies, Malaysia) (12).

Extraction of *G. lucidum* triterpenoids (GLTs)

The fruiting bodies powders (1 kg) were extracted with ethanol 70% (1:10, w/v) in a shaker at room temperature for 24 hours. The extract was filtered firstly by gauze cloth then by Whatman filter paper number 1. The resulting filtrate was evaporated to dryness at 35 ºC under a vacuum using a rotary evaporator (Heidolph, Germany), to produce the concentrated ethanolic extract. The concentrated ethanolic extract was extracted with chloroform and water (1:1 v/v) three times. The chloroform layer was extracted with 5% saturated aqueous $NaHCO_3$ three times. $NaHCO_3$ layer was collected and acidified to pH 3-4 with 2N HCl under ice-cooling and re-extracted with chloroform. The chloroform was evaporated under a rotary evaporator and then transferred to small beaker to ensure evaporate all chloroform. The remaining mixture of acidic compounds is *Ganoderma lucidum* triterpenoids (GLTs). This was stored in a dark airtight container in a refrigerator until used (13).

HPLC analysis of GLTs

HPLC model SYKAM (Germany) was used to analyze GLTs and detection of Ganoderic Acid by using (Ganoderic Acid A) as a standard (ChemFaces company, China. Purity >=98%). The mobile phase was ethanol : acetic acid 5% (40 : 60 v/v). The flow rate was 1.0 ml/min, the column was C18–ODS (25 cm * 4.6 mm), the injection volume was 0.1 ml, and the detector UV-Vis was set at 243 nm (14).

Extraction of *G. lucidum* polysaccharides (GLPs)

The fruit bodies powders (1 kg) were soaked overnight at room temperature in 70% ethanol, (this process was performed to remove low molecular weight materials and lipids). The fruit bodies residues were collected by gauze, then extracted twice from hot water with 30 times water (water/material) in a shaking water bath (Memmert, Germany), at 75 °C with 40 rpm for 2 h. The suspension was subjected to filtration by gauze then Whatman filter paper no. 1. The supernatant was collected and concentrated by using a rotary evaporator at 60 °C. The concentrated water extract was precipitated by 80% ethanol overnight (the volume of 80% ethanol was three times the volume of the concentrated water extract). The next day, polysaccharides were precipitated, and the precipitate was collected by centrifugation at 10000 rpm for 20 minutes. The residue was dried at 45 °C for 24 h, and the resulting dried precipitate is *G. lucidum* polysaccharides (GLPs). It was kept at 4 °C until use (15).

HPLC analysis of GLPs

An auto sampler high-performance liquid chromatography system (Sykam, Germany) equipped with a C18-NH analysis column (250 mm x 4.6 mm, 5 μm) was utilized for the analysis. The composition of the mobile phase was distilled water to methanol at a ratio of 98:2.The flow rate was 0.7 mL/min, and the injected volume was 100 µL, with the detection using a refractive index detector (RI) (16).

Collection of pathogenic bacteria

The pathogenic bacteria were isolated from two hospitals in Baghdad, Al-Imam Ali General Hospital and Ghazy Al-Hariri Hospital for Surgical Specialties, Medical City. 227 samples were collected from the mentioned hospitals. The pathogenic bacteria were isolated from different samples including urine, ear swabs, sputum, stool, wounds and throat swabs in different ages of both genders. Multiple enriched and selective media were used to isolate the pathogenic bacteria such as MacConkey agar, Blood agar, Mannitol salt agar, and Chocolate agar. These bacteria were incubated at 37 ˚C for 24 hours and identified according to the morphological characteristics and microscopic examinations. Plates that did not show growth after 24 hours were considered to be negative samples (17, 18).

Antibiotic susceptibility test

The clinical bacterial isolates were tested for antibiotic susceptibility according to the guidelines of CLSI, 34^{th} Edition, 2024. This guideline was applied to classify the bacteria as either sensitive (S), intermediate (I), or resistant (R) to the recommended antibiotics. This experiment was carried out through the disc diffusion method according to Bauer and Kirby's method in a basic procedure. Following inoculation of the plates of Muller Hinton Agar by the bacterial suspensions, the discs of the recommended antibiotics were put on the surface of the inoculated plates and incubated at 37°C for 18 to 24 hours. After that, the inhibition zone diameter in millimeters was measured around each antibiotic disc, representing the bacterial susceptibility for each antibiotic (19, 20).

VITEK-2 compact system

The four MDR bacterial isolates used in this study were identified, and the species were approved using the VITEK-2 compact system with several biochemical tests (21).

Antibacterial activity of GLTs and GLPs

Five concentrations from each of GLTs and GLPs were used to evaluate the antibacterial efficacy: 6.25, 12.5, 25, 50 and 100 mg/ml using DMSO for GLTs and deionized distilled water for GLPs as a solvent. The agar well diffusion method mentioned by (22) was employed to conduct this experiment in a basic procedure: two to four colonies were collected from bacterial isolates and were suspended in 5 ml normal saline (in plain tubes 10 ml), and calibrated the turbidity of the suspension with McFarland standard 0.5 (1.5×10^8 cfu/ml). The bacterial suspensions were distributed according to the Lawn Culture Method, by using sterile cotton swabs onto the surface of Muller Hinton Agar (MHA), by turning the plates three times to distribute the bacteria evenly across the surface of the agar. The inoculated plates were left for 20 minutes to absorb the moisture. A hole with 5 mm diameter was punched using a sterile cork borer, and then 60 µL from the mentioned concentrations were introduced into each well (each plate had five wells with five concentrations). All plates were sealed around the whole circumference with laboratory para-film. The plates were incubated for 24 hours at 37°C. The diameter of the inhibitory zone, measured in millimeters, represents the antibacterial activity of each concentration; each treatment has consisted of three replicates (23).

Statistical analysis

The statistical analysis was performed using the SPSS-25 program for data analysis according to the two-way analysis of variance (Tow –Way ANOVA). A complete randomized design (CRD) was established with three replicates per treatment. The means were separated using the Least Significant Differences test (LSD) and P value of ≤ 0.05.

Results and Discussions

Extraction of GLTs

From the 1000 g of *G. lucidum* dried fruiting bodies powder which were used to extract the triterpenoids, 16.1 g GLTs were extracted. This translates to 1.61% of the total powder. This result agreed with the study of (24) which extracted 1.67% of *G. lucidum* powder. However, this result did not agree with the study of (13) which extracted 2.27% of GLTs from fruiting boding powder.

HPLC analysis of GLTs

The HPLC method was established by comparing the retention times of GLTs peak with the Ganoderic acid (A) standard. The results in Figure (1) demonstrated the same peaks between the sample and the standard with the identical retention time (6.25 mins). The results demonstrated that the concentration of Ganoderic acid in the sample was 985 µg per gram of the extract.

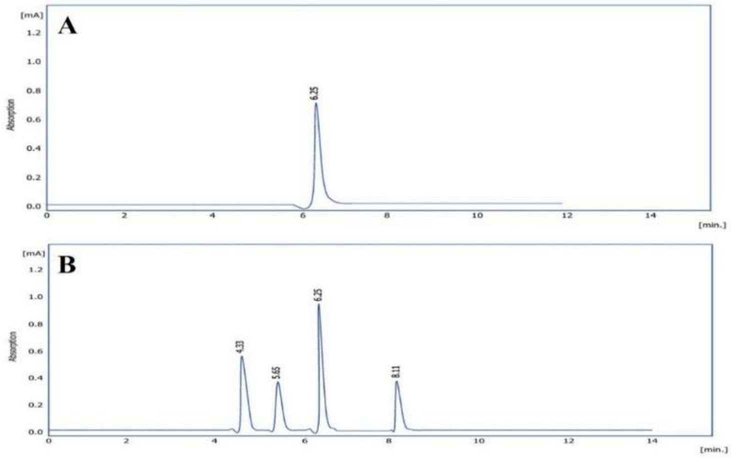

Figure (1): HPLC chromatogram: (A): Ganoderic Acid A standard. (B): GLTs sample.

Extraction of GLPs

About 1000 g of the dried fruiting bodies powder which were used to extract the polysaccharides, 15.7 g GLPs was extracted. This translates to 1.57% of the total powder.

HPLC analysis of GLPs

The HPLC method was established by comparing the retention times of the GLPs peak with β-Glucan standard. The results in Figure (2) showed the same peaks between the sample and the standard with identical retention time (5.25 mins). The results demonstrated that the concentration of β-Glucan in the sample was 416.25 µg per gram of the extract.

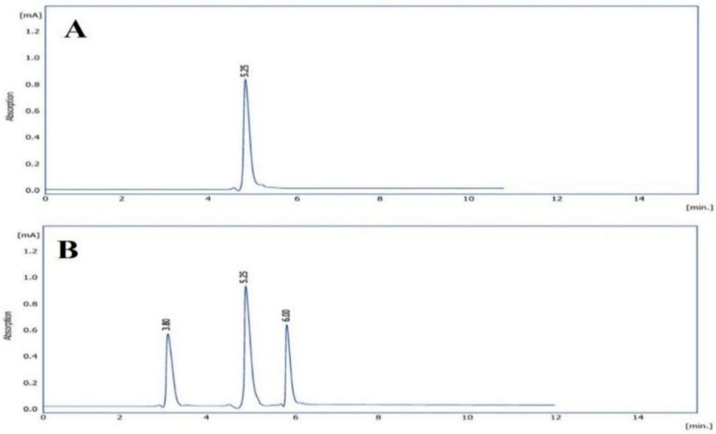

Figure (2): HPLC chromatogram: (A): β-Glucan standard. (B): GLPs sample.

Antibiotic susceptibility test

The clinical bacteria were examined for antibiotic sensitivity by disc diffusion method according to the guideline of CLSI, 2024. From 135 positive bacterial samples, four isolates were selected which showed MDR. In other words, these four bacteria exhibited resistance to nearly all tested antibiotics, with the exception of some antibiotics which were sensitive (Table 1).

Table (1): The result of antibiotic susceptibility testing for the selected bacteria (R: resistance, S: sensitive).

Antibiotic	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. faecalis*
Ampicillin	R	R	R	S
Azithromycin			R	R
Gentamycin	R	R	R	R
Vancomycin			S	S
Ciprofloxacin	R	R	R	R
Levofloxacin	R	R	S	S
Erythromycin	R			R
Tetracycline	R	R		R
Ceftazidime	R	R	R	R
Cefepime	R	R		
Trimethoprim	R		R	
Imipenem	S	R		
Ceftriaxone	R	R	S	R
Amikacin	S		R	R
Meropenem	S	R	R	

VITEK-2 compact system

The VITEK2 compact system improved the species of bacteria which were mentioned above, all bacteria had 99% probability (Table 2).

Table (2): Identification information of VITEK-2 for the MDR selected bacteria

Selected organism	Probability	Bionumber	Analysis time (hours)
Enterococcus faecalis	99%	116012761753471	3.12
Staphylococcus aureus	99%	010402062763031	3.83
Escherichia coli	99%	0405610454426611	3.38
Pseudomonas aeruginosa	99%	6222649203427140	16.8

Antibacterial activity of GLTs and GLPs

The antibacterial activity of the above extracts was studied against MDR Gram-negative bacteria (*P. aeruginosa* and *E. coli*) and gram Gram-positive bacteria (*S. aureus* and *E. faecalis*). Five concentrations were used from each extract: 6.25, 12.5, 25, 50 and 100 mg/ml.

For GLTs, the mean inhibition zone for *E. faecalis* and *S. aureus* was lower at a concentration of 12.5 mg/ml and higher at a concentration of 100 mg/ml. The mean inhibition zone for *E. coli* was lower at a concentration of 50 mg/ml and higher at a concentration of 100 mg/ml. The mean inhibition zone for *P. aeruginosa* was lower at a concentration of 25 mg/ml and higher at a concentration of 100 mg/ml (Table 3).

The concentration of 100 mg/ml of GLTs exhibited the most substantial inhibitory zones against bacteria, measuring 16.33 mm, 19.33 mm, 13 mm, and 15.33 mm for *E. faecalis*, *S. aureus*, *E. coli*, and *P. aeruginosa* respectively. In another words, the inhibition zone increased with increasing the concentrations (Figure 3).

Table (3): The inhibition zone (millimeter) of MDR bacterial isolates by using GLTs at (6.25, 12.5, 25, 50, and 100 mg/ml) concentrations on Muller Hinton Agar during 24 hours of incubation at 37˚C (each treatment consisted of three replicates).

Bacteria	Inhibition Zone (mm) GLTs concentrations (mg/ml)					Ceftriaxone	Control	Mean
	6.25	12.5	25	50	100			
E. faecalis	0.00	10.00	11.66	14.00	16.33	0.00	0.00	7.42
S. aureus	0.00	6.00	12.66	14.33	19.33	9.66	0.00	8.85
E. coli	0.00	0.00	0.00	9.66	13.00	0.00	0.00	3.23
P. aeruginosa	0.00	0.00	8.00	10.33	15.33	0.00	0.00	4.80
Mean	0.00	4.00	8.08	12.08	15.99	2.41	0.00	
L S D	$P \leq 0.05$							

In case of GLPs, the mean inhibition zone for *E. faecalis* was lower at a concentration of 50 mg/ml and higher at a concentration of 100 mg/ml. The mean inhibition zone for *S. aureus* was lower at a concentration of 12.5 mg/ml and higher at a concentration of 100 mg/ml. The mean inhibition zone for *E. coli* and *P. aeruginosa* was lower at a concentration of 25 mg/ml and higher at a concentration of 100 mg/ml (Table 4).

The concentration of 100 mg/ml of GLPs significantly inhibited all examined bacteria, yielding the maximum inhibition zones of 15 mm for *E. faecalis*, 19.66 mm for *S. aureus*, 13.33 mm for *E. coli*, and 14.33 mm for *P. aeruginosa* (Figure 3).

On the other hand, the positive control (Ceftriaxone antibiotic) exhibited no inhibition zone on all tested bacteria, except for *S. aureus* which revealed a 9.66 mm zone of inhibition. The negative control (deionized distilled water for GLPs and DMSO for GLTs), showed no inhibition zone on all examined bacteria.

Table (4): The inhibition zone (millimeter) of MDR bacterial isolates by using GLPs at (6.25, 12.5, 25, 50, and 100 mg/ml) concentrations on Muller Hinton Agar during 24 hours of incubation at 37˚C (each treatment consisted of three replicates).

Bacteria	Inhibition Zone (mm) GLPs concentrations (mg/ml)					Ceftriaxone	Control	Mean
	6.25	12.5	25	50	100			
E. faecalis	0.00	0.00	0.00	10.00	15.00	0.00	0.00	3.57
S. aureus	0.00	11.00	11.66	16.00	19.66	9.66	0.00	9.71
E. coli	0.00	0.00	5.33	8.66	13.33	0.00	0.00	3.90
P. aeruginosa	0.00	0.00	6.00	11.00	14.33	0.00	0.00	4.47
Mean	0.00	2.75	5.74	11.41	15.58	2.41	0.00	
L S D	$P \leq 0.05$							

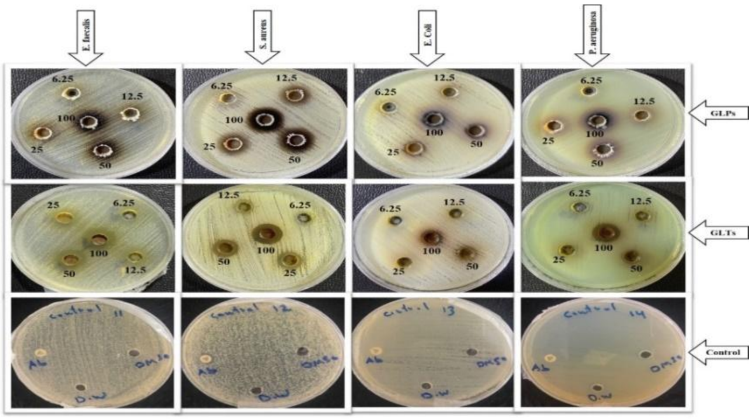

Figure (3): The antibacterial activity of GLPs and GLTs against bacterial isolates at (6.25, 12.5, 25, 50, and 100 mg/ml) concentrations compared with negative control and ceftriaxone antibiotic.

In this study, the antibacterial activity of the above extracts was studied against MDR bacteria. These extracts have higher antibacterial activity against all bacteria.

Antibiotic resistance is developing in many organisms, and multidrug-resistant organisms provide significant risks to the treatment of infections. Consequently, antimicrobial compounds produced from mushrooms received significant attention in the past few years. The present study indicates that *G. lucidum* mushroom extracts may be utilized to address various disorders caused by pathogenic bacteria (25, 26). The aim of natural antimicrobial compounds is primarily to kill the microorganism, inhibit their proliferation without damaging normal cells, and preserve against microbial resistance. *G. lucidum* extracts exhibit extensive antibacterial properties, targeting both Gram-positive and Gram-negative bacteria. The majority of studies have focused on the crude extracts of *G. lucidum*, with limited studies targeting polysaccharides and triterpenoids, which are the primary active components of this mushroom. Typically, *G. lucidum* extracts using water and several organic solvents are utilized against multiple gram positive and negative bacteria (25).

Polysaccharides derived from fungi, particularly those from edible and medicinal Basidiomycetes, exhibit significant immunomodulatory, antioxidant, and antimicrobial properties. Despite the extensive efforts of numerous researchers to produce bioactive metabolites from mushrooms. Many researchers have examined the antibacterial properties of medicinal mushrooms. Certain mushroom extracts have been documented to have antimicrobial action against different resistance bacteria (27), supporting the findings of this study. (28) examined the chloroform extract isolated from the fruiting body of *G. lucidum*. They discovered that this extract exhibited antibacterial properties against G+ve bacteria *Bacillus subtilis, Staphylococcus aureus*, *Enterococcus feacalis*, and G-ve bacteria *Escherichia coli*, *Pseudomonas aeruginosa*. In addition, research conducted by (29) demonstrated that certain extracts of *G. lucidum* exhibit a greater antibacterial activity compared to the traditional antibiotics. Consequently, this medicinal mushroom has the ability to impede the progression of many bacterial infections.

Comprehensive investigations conducted to clarify the inhibitory mechanism of *G. lucidum* extractions revealed the production of powerful reactive oxygen species (ROS) within the intracellular environment of bacterial pathogens. Previous researchers have identified the formation of reactive oxygen species and other oxidative agents and may be a primary mechanism of bacterial cell death (30).

Conclusions

Ganoderma lucidum is one of the most important medicinal mushroom due to has several active ingrediants. GLTs and GLPs were successfully extracted and identified from this medicinal mushroom. These extracts indicated enhanced bactericidal efficacy against antibiotic resistance bacteria (two Gram-negative and two Gram-positive bacteria). These results suggest that both triterpenoids and polysaccharides extracted from *G. lucidum* mushroom possess promising antibacterial properties against MDR bacteria and demand further research to clarify their mechanisms of action and investigate their potential as novel therapeutic agents.

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