iJRASET
International Journal For Research in Applied Science and Engineering Technology



INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 6 **Issue:** VII **Month of publication:** July 2018

DOI: http://doi.org/10.22214/ijraset.2018.7060

www.ijraset.com

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 6.887
Volume 6 Issue VII, July 2018- Available at www.ijraset.com

Antimicrobial Activity of Extracellular Proteins Secreted by *Bacillus subtilis* ATCC21332 after being Induced with *Cymbopogon flexuosus* Essential Oil and Cultured in Different Growth Conditions

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Abstract: ***Extracellular proteins secreted by diverse bacteria with broad spectrum of antimicrobial activity can be used as an alternative treatment against antibiotic resistant and spoilage microorganisms. However, the extracellular proteins produced by bacteria are affected by environmental stress factors and growth conditions. Therefore, antimicrobial activity of extracellular proteins secreted by Bacillus subtilis ATCC21332 in the presence of Cymbopogon flexuosus as stress inducer and cultivated in different growth conditions was evaluated against four selected strains of Gram-positive and Gram-negative bacteria, including Bacillus cereus, Escherichia coli, Shigella sonnei and Staphylococcus epidermidis by agar-well diffusion test and microdilution assay. Agar-well diffusion test was carried out to identify the antimicrobial susceptibility, while microdilution assay was done to determine the inhibition and bactericidal effects. B. subtilis ATCC21332 cells were treated with a low concentration (0.01 MIC) of C. flexuosus essential oil and cultured in various growth conditions such as different pH media (pH 6, pH 7 and pH 8) or nutrient sources, including 1% (w/w) of carbon (glucose, sucrose and starch), 1% (w/w) of nitrogen (casein, gelatin and urea) and 1% (w/w) of inorganic salt (sodium nitrate, calcium chloride and sodium dihydrogen phosphate). After 72 h of fermentation at 30°C, the secreted extracellular proteins were then extracted and analysed for antimicrobial activity. Results showed that the extracellular proteins secreted by stress induced B. subtilis ATCC21332 and cultivated in media with 1% of sucrose exhibited antimicrobial activity against two bacterial strains which are S. epidermidis and B. cereus with 8.0 ± 0.8 mm and 6.0 ± 0.0 mm of inhibition zone respectively. Meanwhile, the extracellular proteins extracted from bacteria cultured in each different media supplemented either with 1% of casein, 1 % of sodium nitrate and 1% of calcium chloride only showed antimicrobial activity against B. cereus with inhibition zone of 7.5 ± 0.5 mm, 8.0 ± 1.0 mm and 9.0 ± 0.0 mm respectively. Although the extracellular proteins indicated inhibitory effect against B. cereus and S. epidermidis, however the proteins only exhibited bactericidal effect towards B. cereus.***

Keywords: ***Antimicrobial Activity, Extracellular Proteins, Bacillus subtilis ATCC21332, Cymbopogon flexuosus Essential Oil.***

I. INTRODUCTION

Multidrug-resistance against pathogenic bacteria has been increased alarmingly during the last few decades due to the over usage. Extensive efforts have focused on the development of new classes of antimicrobial agents with novel modes of action and more effective killing [1]. Therefore, it is important to identify different classes of compounds and recover natural molecules to gain new lead compounds with potential therapeutic use [2].

In recent years, antimicrobial proteins or peptides (AMPs) are gaining greater attention as alternative therapeutics against antibiotic resistant pathogens and spoilage bacteria. These natural compounds possess broad-spectrum of antimicrobial activity towards pathogenic microorganisms includes Gram-negative and Gram-positive bacteria, filamentous fungi as well as parasitic protozoa and metazoan [3]-[4]. Production of AMPs is widespread among diverse bacteria. For instance, *Bacillus* sp. produce a broad spectrum of bioactive protein or peptides with great potential for biotechnological and biopharmaceutical applications [5]-[6].

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Bacterial cells are constantly exposed to any environment that may reduce their viability. They will launch adaptive responses to survive when facing constant changes in environments such as changes in external temperature, pH and nutrient supply as well as osmolarity imbalances and rising of oxidative stress [7]. These conditions may activate bacterial adaptive mechanism as a response in order to protect the cell from fatal environment factors as well as to repair the damage caused by the harsh condition [8]. As a result, a new protein or high yield of certain protein will be produced or secreted by bacterial cells in these stress environments [9].

Antimicrobial agents are bioactive compounds that can serve as weapon due to their inhibitory activity toward other microorganisms. In ecological environments, these compounds may be at lower concentrations and likely play additional roles as signalling molecules [10]. It appears that many antimicrobial agents, when used at low concentrations, have the ability to activate gene transcription, which is distinct from their inhibitory effect [11]. For example, the antimicrobial agents at sub-minimal inhibitory concentration (sub-MICs) were found to enhance and modulate the production of new phenazines, streptophenazines A-H, in a marine *Streptomyces* isolate. Streptophenazines showed an antimicrobial activity against *B. subtilis* and *S. lentus* [12].

Cymbopogon flexuosus essential oil is known as source of natural citral that mainly composed of geranial and neral [13]. The oil exhibited antimicrobial activity towards foodborne and pathogenic bacteria [14]-[15]. Besides, it also showed post-antibiotic effects (PAE) against *S. aureus* and *E. coli* [16]. However, at low minimal inhibitory concentration (MIC) the oil could induce *B. subtilis* ATCC21332 to produce new protein recognized as Bacillopeptidase F [17]. In addition, it was found that *C. flexuosus* essential oil at low concentration could act as signalling molecules with the ability to induce global changes in gene transcription or gene expression [18].

The discovery of new antibacterial agents in recent years has increases due to rise of life-threatening infections by antibiotic-resistant bacteria [19]. Antimicrobial substances at low concentration could act as chemical signals to modulate metabolic processes in bacteria in which may activate signal transduction cascades that result in the production of bioactive proteins [10]. The production of bioactive proteins by bacterial cells is highly influenced by many factors such as pH, temperature, incubation period, cell density and nutrient sources [6]. Therefore, the study of environmental stress factors and growth conditions is very important in order to enhance the production of bioactive protein by microbes in which may be further used for antimicrobial therapy. Attempt has been made to determine the antimicrobial activity of extracellular proteins produced by Bacillus sp. during mild stress condition in the presence of *C. flexuosus* essential oil and changes of growth condition.

II. MATERIALS AND METHODS

A. Essential Oils, Bacterial Strains and Culture Conditions

C. flexuosus essential oil used as stress inducer was provided by Al-muqarram Holdings Sdn. Bhd. *B. subtilis* ATCC21332 cells, obtained from American Type Culture Collection (ATCC) were cultured in Nutrient Broth (NB; Merck, Germany) and maintained on Nutrient Agar (NA; Merck, Germany). Test microorganisms used in antimicrobial screening include *B. cereus, S. epidermidis, E. coli* and *S. sonneii*, obtained from Microbiology Laboratory, Faculty of Science and Technology, USIM were grown in Mueller Hinton Broth (MHB; Oxoid, USA).

B. Microbial Protein Production

Microbial protein production was done by varying one-parameter-at-a-time approach, based on method by Vijayaraghavan et al. (2014) and Hanina et al. (2014) [20], [17]. *B. subtilis* ATCC21332 cells were treated with 0.01 MIC of *C. flexuosus* essential oil and cultured in different pH media (pH 6, pH 7 and pH 8) or variable of nutrient sources, including 1% (w/w) of carbon (glucose, sucrose and starch), 1% (w/w) of nitrogen (casein, gelatin and urea) and 1% (w/w) of inorganic salt (sodium nitrate, calcium chloride and sodium dihydrogen phosphate) before being further incubated at 30°C for 72 h of fermentation with agitation using shaker incubator (Sastec, Malaysia). Bacterial cells grow in the presence of *C. flexuosus* essential oil as stress inducer at normal pH media without addition of nutrients were served as control.

C. Microbial Protein Extraction

Microbial protein extraction was done based on method by Singh et al. (2012) [21]. After 72 h of fermentation process, each bacterial culture was spin at 10,000 x g for 10 min at 4°C using centrifuge (Hanil, Combi 514R, Korea) to separate the supernatant from pellet containing bacterial cells. The supernatant was then filtered using 0.45 µm filter (Millipore, USA). The filtrate containing extracellular protein was ready for antimicrobial screening.

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ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 6.887
Volume 6 Issue VII, July 2018- Available at www.ijraset.com

D. Antimicrobial Activity Screening via Well Diffusion Test and Microdilution Assay

Antimicrobial activity of extracellular protein secreted by *Bacillus subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil with various parameter of growth conditions was evaluated against *B. cereus*, *S. epidermidis*, *E. coli* and *S. sonneii* by agar-well diffusion assay and microdilution test. Screening of antimicrobial susceptibility via agar-well diffusion test was done according to method by Singh et al. (2012) [21]. Each selected bacterial culture at concentration of 10^6 cells / ml was spread onto Mueller-Hinton Agar (MHA, Merck, Germany) plates. Wells with 6 mm of diameter were made on agar by using sterile borer. A 100 µl of each isolated microbial protein and control samples were loaded into each test well. The agar plates were incubated at 37°C and the diameter of the inhibition zones were measured after 24 h of incubation. The microdilution assay was done based on method by Balouiri et al. (2016) [22] to determine the inhibitory effect and bactericidal activity. A 100 µl of bacterial inoculum at concentration of 10^6 cell/ml were added into each wells of sterilized 96-well microtiter plate containing 100 µl of isolated microbial protein and control samples, before being further incubated at 37°C for 24 h. The turbidity of test and control samples was observed before being streaked on MHA plates. The bacterial regrowth was observed and evaluated after incubation at 37°C for 24 h.

III. RESULTS AND DISCUSSION

Study on environmental stress factors and growth conditions are important in order to increase the bioactive protein production by microbes in which may be further used for antimicrobial therapy. In this study, *B. subtilis* ATCC21332 cells were treated with 0.01 MIC of *C. flexuosus* essential oil and cultured in different pH media (pH 6, pH 7 and pH 8) or variable of nutrient sources, including 1% (w/w) of carbon (glucose, sucrose and starch); 1% (w/w) of nitrogen (casein, gelatin and urea) and 1% (w/w) of inorganic salt (sodium nitrate, calcium chloride and sodium dihydrogen phosphate). The extracellular proteins secreted by bacterial cells were then extracted and examined for its antimicrobial activity via agar-well diffusion test and microdilution assay. As a control, the extracellular proteins produced by bacterial cells in the presence of *C. flexuosus* essential oil and grow in normal pH without media modification was also study for antimicrobial activity. The antimicrobial activity of extracellular proteins was evaluated against selected four Gram-positive and Gram-negative bacterial strains, including *B. cereus*, *S. epidermidis*, *E. coli* and *S. sonnei*.

The spectrum of antimicrobial activity via agar-well diffusion test was determined based on the size of inhibition zone formed as shown in Fig. 1 and summarized in Table 1. The extracellular proteins secreted by *B. subtilis* ATCC21332 prior to treatment with 0.01 MIC of *C. flexuosus* essential oil and grown in media with 1% of sucrose showed antimicrobial activity towards two Gram-positive bacterial strains which are *S. epidermidis* and *B. cereus* with each size of inhibition zone 8.0 ± 0.8 mm and 6.0 ± 0.0 mm respectively. Meanwhile, the extracellular proteins produced by *B. subtilis* ATCC21332 after being treated with 0.01 MIC of *C. flexuosus* essential oil and supplied with either 1% of casein or 1 % of sodium nitrate or 1% of calcium chloride only showed antimicrobial activity against *B. cereus* with inhibition zone of 7.5 ± 0.5 mm, 8.0 ± 1.0 mm and 9.0 ± 0.0 respectively.

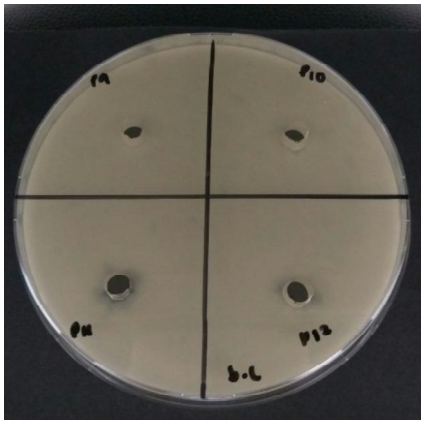


Fig. 1 The Formation of Inhibition Zone by Agar-Wells Diffusion Test.
Note: Formation of clear inhibition zone which represent existence of antimicrobial activity of the test samples.

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However, the extracellular proteins synthesized by *B. subtilis* ATCC21332 prior to treatment with 0.01 MIC of *C. flexuosus* essential oil and cultured in different pH media or supplied with variable nutrients other than sucrose, casein, sodium nitrate and calcium chloride did not indicated any antimicrobial activity towards all bacterial tests. The control protein sample (C) which is the protein synthesized by *B. subtilis* ATCC21332 after inducing with 0.01 MIC of *C. flexuosus* essential oil without media modification also did not showed any antimicrobial activity. Generally, Gram-positive bacteria are easily to be inhibited compared to Gram-negative bacteria. The Gram-negative organisms are less susceptible to the action of antimicrobials since they possess an outer layer of membrane which protects the cell wall. The cell wall restricts the hydrophobic compounds to diffuse through the lipopolysaccharide covering [23].

TABLE 1
Spectrum of Antimicrobial Activity of Proteins Produced by *B. subtilis* ATCC21332

	Test/Control Samples	Test Microorganisms			
		B. Cereus	*S. epidermidis*	*E. coli*	*S. Sonnei*
[a]pH media	pH 6	[g]-	-	-	-
	pH 7	-	-	-	-
	pH 8	-	-	-	-
[b]Carbon Source	Sucrose	6.0 ± 0.0 mm	[h]8.0 ± 0.8 mm	-	-
	Starch	-	-	-	-
	Glucose	-	-	-	-
[c]Nitrogen Source	Urea	-	-	-	-
	Casein	7.5 ± 0.5 mm	-	-	-
	Gelatin	-	-	-	-
[d]Inorganic Salts	Sodium dihydrogen phosphate	-	-	-	-
	Sodium Nitrate	8.0 ± 1.0 mm	-	-	-
	Calcium chloride	9.0 ± 0.0 mm	-	-	-
[e]C		-	-	-	-
[f]S		25.7 ± 0.5 mm	-	22.3 ± 1.3 mm	26.3 ± 1.3 mm

Notes: [a]Protein produced by *B. subtilis* ATCC21332 with media pH adjustment in the presence of 0.01 MIC of *C. flexuosus* essential oil; [b]Protein produced by *B. subtilis* ATCC21332 with 1% of carbon sources in the presence of 0.01 MIC of *C. flexuosus* essential oil; [c]Protein produced by *B. subtilis* ATCC21332 with 1% of nitrogen sources in the presence of 0.01 MIC of *C. flexuosus* essential oil; [d]Protein produced by *B. subtilis* ATCC21332 with 1% of inorganic salts in the presence of 0.01 MIC of *C. flexuosus* essential oil; [e]C Protein produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil without modification of growth conditions; [f]Streptomycin sulphate as a positive control; [g]- No inhibition zone = No antimicrobial activity; [h]+ Inhibition zone = Has antimicrobial activity.

Inhibitory effect and bactericidal activity of extracellular proteins secreted by *B. subtilis* ATCC21332 in the presence of 0.01 MIC of *C. flexuosus* essential oil with modification of media were also evaluated using microdilution assay. The microdilution assay is more effective than well diffusion test as the samples have direct contact with the test microorganisms [24]. Moreover, the antimicrobial study by agar-well diffusion test may also be affected by certain factors such as volume of bacteria inoculates, culture media selected, growth phase, incubation time, temperature subjected and the ability of test sample to dissolve in agar medium [25]-[26].

The results of inhibitory effect for all extracellular protein samples and control sample produced by *B. subtilis* ATCC21332 at 30°C for 72 h of fermentation process are summarized in Table 2. Generally, the extracellular proteins secreted by *B. subtilis* ATCC21332 after being treated with 0.01 MIC of *C. flexuosus* essential oil and cultured in different pH media or supplied with variable nutrients could only inhibit *B. cereus*. However, the extracellular proteins produced by *B. subtilis* ATCC21332 prior to treatment with 0.01 MIC of *C. flexuosus* essential oil and grown in media with 1% of sucrose showed inhibitory effect against two Gram-positive bacterial strains which are *B. cereus* and *S. epidermidis*. Besides, the control protein sample (C) which is the protein

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synthesized by *B. subtilis* ATCC21332 after inducing with 0.01 MIC of *C. flexuosus* essential oil without media modification also indicated inhibitory effect towards *B. cereus*. It showed that the presence of *C. flexuosus* essential oil at low concentration (0.01 MIC) without medium modification still induce the production of bioactive protein with antimicrobial activity. Previous study has been reported that the protein produced by *B. subtilis* ATCC21332 after inducing with 0.01 MIC of *C. flexuosus* essential oil for 48 h of fermentation at 30°C exhibited inhibitory activity against *B. cereus* [17].

TABLE 2
Inhibitory Effect of Proteins Produced by *B. subtilis* ATCC21332

	Test/Control Samples	Test Microorganisms			
		B. cereus	*S. epidermidis*	*E. coli*	*S. Sonnei*
[a]pH media	pH 6	[g]+	[h]-	-	-
	pH 7	+	-	-	-
	pH 8	+	-	-	-
[b]Carbon Source	Sucrose	+	+	-	-
	Starch	+	-	-	-
	Glucose	+	-	-	-
[c]Nitrogen Source	Urea	+	-	-	-
	Casein	+	-	-	-
	Gelatin	+	-	-	-
[d]Inorganic Salts	Sodium dihydrogen phosphate	+	-	-	-
	Sodium nitrate	+	-	-	-
	Calcium chloride	+	-	-	-
[e]C		+	-	-	-
[f]S		+	+	+	+

Notes: [a]Protein produced by *B. subtilis* ATCC21332 with media pH adjustment in the presence of 0.01 MIC of *C. flexuosus* essential oil; [b]Protein produced by *B. subtilis* ATCC21332 with 1% of carbon sources in the presence of 0.01 MIC of *C. flexuosus* essential oil; [c]Protein produced by *B. subtilis* ATCC21332 with 1% of nitrogen sources in the presence of 0.01 MIC of *C. flexuosus* essential oil; [d]Protein produced by *B. subtilis* ATCC21332 with 1% of inorganic salts in the presence of 0.01 MIC of *C. flexuosus* essential oil; [e]C Protein produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil without modification of growth conditions; [f]Streptomycin sulphate as a positive control; [g]+ No bacterial growth (non-turbid) = Has inhibition effect [h]- Bacterial growth (turbid) = No inhibition effect.

The bactericidal activity was determined by inoculating each of protein and control samples with test microorganisms and incubated for 24 h at 37°C before being streaked on MHA. The MHA plates were incubated at 37°C for another 24 h to allow further growth of bacteria as shown in Fig. 2. The result of bacterial regrowth considered as bacteriostatic, while the result of no bacterial regrowth showed bactericidal effect [27]. The bacteriostatic effect indicated that the extracellular proteins could only inhibit the bacterial test. Whilst, the bactericidal effect showed that the extracellular proteins could kill the bacterial test. The results of bactericidal activity as shown in Table 3 are quite similar with the results of inhibitory effect. In this experiment, the extracellular proteins produced by *B. subtilis* ATCC21332 prior to treatment with 0.01 MIC of *C. flexuosus* essential oil and cultured in different pH media (except pH 8) or supplied with variable nutrients (excluding 1 % of glucose) only showed bactericidal activity against *B. cereus*. The Gram-positive bacteria can be easily inhibited compared to Gram-negative bacteria. It has been reported that the Gram-

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negative bacteria could resist to microbicidal substance such as antimicrobial agents due to its protective outer membrane porins in which can continuously alter its expression and functions. [28].



Fig. 2 Analysis of Bactericidal and Bacteriostatic Activities by Microdilution Assay.
Notes: The result of bacterial regrowth considered as bacteriostatic, while the result of no bacterial regrowth showed bactericidal effect.

TABLE 3
Bactericidal and Bacteriostatic Effect of Proteins Produced by *B. subtilis* ATCC21332

	Test/Control Samples	Test Microorganisms			
		B. cereus	*S. epidermidis*	*E. coli*	*S. sonnei*
[a]pH media	pH 6	[g]+	[h]-	-	-
	pH 7	+	-	-	-
	pH 8	-	-	-	-
[b]Carbon Source	Sucrose	+	-	-	-
	Starch	+	-	-	-
	Glucose	-	-	-	-
[c]Nitrogen Source	Urea	+	-	-	-
	Casein	+	-	-	-
	Gelatin	+	-	-	-
[d]Inorganic Salts	Sodium dihydrogen phosphate	+	-	-	-
	Sodium nitrate	+	-	-	-
	Calcium chloride	+	-	-	-
[e]C		+	-	+	-
[f]S		+	+	+	+

Notes: [a]Protein produced by *B. subtilis* ATCC21332 with media pH adjustment in the presence of 0.01 MIC of *C. flexuosus* essential oil; [b]Protein produced by *B. subtilis* ATCC21332 with 1% of carbon sources in the presence of 0.01 MIC of *C. flexuosus* essential oil; [c]Protein produced by *B. subtilis* ATCC21332 with 1% of nitrogen sources in the presence of 0.01 MIC of *C. flexuosus* essential oil; [d]Protein produced by *B. subtilis* ATCC21332 with 1% of inorganic salts in the presence of 0.01 MIC of *C. flexuosus* essential oil; [e]C Protein produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil without modification of growth conditions; [f]Streptomycin sulphate as a positive control; [g]+ No bacterial regrowth = Has bactericidal effect; [h]- Bacterial regrowth = Has bacteriostatic effect.

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IV. CONCLUSIONS

B. subtilis ATCC21332 in stressful condition in low concentration (0.01 MIC) of *C. flexuosus* essential oil with media modification could induce the production of bioactive protein with antimicrobial activity.

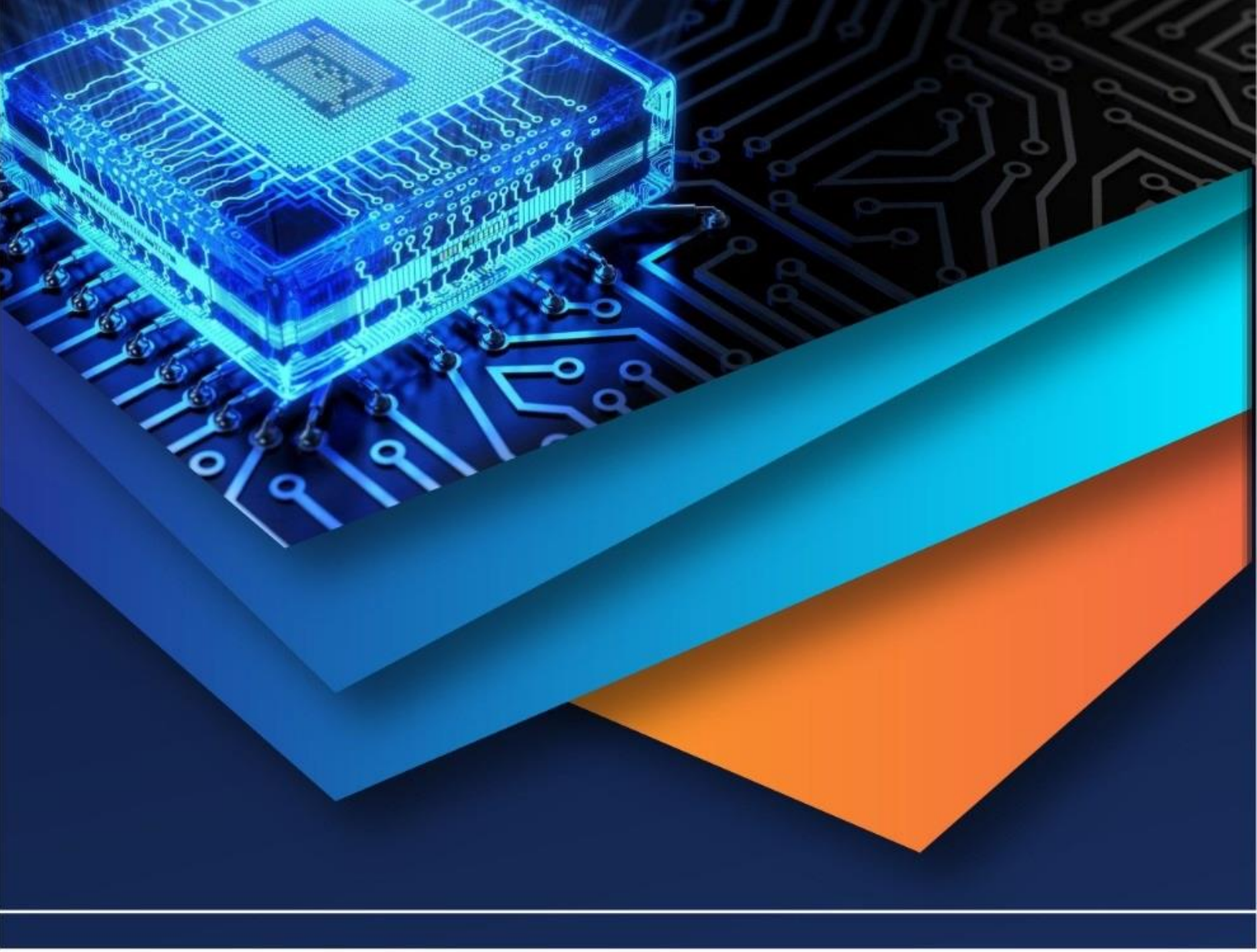
V. ACKNOWLEDGMENT

The authors would like to thank the Ministry of Higher Education of Malaysia for the financial support through FRGS Grant Scheme USIM/FRGS/FST/32/51915 or FRGS/1/2015/SG05/USIM/02/1 and facilities through Universiti Sains Islam Malaysia.

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