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Evaluation of Antimicrobial Susceptibility of the Pigment by Fusarium chlamydosporum against Emerging Nosocomial and Opportunistic Pathogens

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Abstract: ***The present study was carried out to evaluate the antimicrobial efficacy of the pigment produced by the fungus, Fusarium chlamydosporum against the emerging Nosocomial and Opportunistic pathogens which were procured from the American Type Culture Collections; Staphylococcus aureus ATCC®1708™ (Methicillin Resistant) {MRSA), Escherichia coli ATCC®2326™(Extended Spectrum BetaLactamase){ESBL}, Pseudomonas aeruginosa ATCC®27853™, and Acinetobacter baumanii ATCC®19606™, Serratia marcescens ATCC®14756™, and Candida albicans ATCC®10231™. The fungus was procured from the National Fungal Culture Collection Institute (NFCCI), Pune with the Accession number, 3020, and was cultivated in an optimised media for its pigment production. The intracellular pigment produced by F. chlamydosporum was extracted using chloroform and subjected for partial purification via Thin Layer Chromatographic technique. The purified extracts of the fungus were assessed for the Minimum Inhibitory Concentration (MIC) using the Kirby Bauer method. Different dilutions of the extract ranging from the concentrations 5000 µg/ml to 9.7 µg/ml were used. Appropriate positive and negative controls were used in the analysis as per the CLSI standards. The pigment exhibited an excellent antimicrobial actiivty against MRSA (MIC at 39 µg/ml), ESBL E. coli (MIC at 625 µg/ml), A. baumanii (MIC at 312.5 µg/ml), S. marcescens (MIC at 39 µg/ml), and C. albicans (MIC at 312.5µg/ml whereas the pigment showed a negative response against P. aeruginosa. These results suggest that the pigment produced by F. chlamydosporum is a potent metabolite which can be used for various applications in the medical field against the emerging drug resistance and Nosocomial pathogens.***

Keywords: Fusarium chlamydosporum**, MRSA, ESBL, Nosocomial pathogens, Minimum Inhibitory Concentration.**

I. INTRODUCTION

The production of synthetic pigments is economically and commercially an advanced technique, despite its disadvantages of being carcinogenic, toxic, and teratogenic. They also owe in causing long-term side effects especially hormonal imbalance and behavioral problems in children. The emergence of natural pigments from various microbial sources has helped to counteract these harmful effects of synthetic colorants. The fact that most of the European countries have banned the use and manufacturing of synthetic dyes have led to the new field of research of producing different kinds of stable, economical, and commercially valuable natural colorants. The Natural pigments can be classified into Microbial pigments and Plant extracts. Until the 19th century which marked the beginning of synthetic dyes, natural organic colorants like curcuma and indigo (plant extracts), carmine (animal extracts) were being used. Although plant extracts show a very good activity in medical, cosmetic, and textile industry, its production is completely dependent on external factors like seasonal supply of raw materials, climate, and availability which in turn leads to batch-to-batch variations in the extracted pigment profile [1]. In this regard, microbial pigments were found to be a better alternative to satisfy the insatiable demands of the industries. Amongst microbes, fungi, algae, and bacteria have been exploited for the commercial production of pigments. The overlaid advantages of microbial pigments over the plant extracts are that they produce pigments in varying shades, and are independent of seasonal changes. They can also be cultivated on cheap substrates with a carbohydrate and nitrogen composition [2]. The production of these pigments can be easily increased in geometric proportions through genetic engineering when compared to the scaling up process in other sources of pigment production [3].

Fungi are known to be the potent producers of secondary metabolites (includes pigments and mycotoxins). They are more suitable for biotechnological production of pigments due to higher yield and easier culture techniques [4]. They prove to be a potential alternative for synthetic compounds in various fields of the industry. Most of the chronic ailments like cancer, diabetes, and other metabolic disorders are associated with free radicals. With regard to these, the different class of pigments like carotenoids,

lycopene, naphthaquinone, etc produced by different fungi or species of fungi have been known to possess antioxidant properties [5]. These pigments also possess the potential to act as antibiotics, anticancer, antiproliferative, and immunosuppressive compounds. Pigments from Fusarium graminearum [6] and Fusarium fujikuroi [7] have been studied. Bikaverin pigment productions by many Fusarium strains and its clinical applications have been carried out [8]. Fusarium chlamydosporum also produces biologically active metabolites, e.g. pigments, mycotoxins, phytotoxins, naphthoquinone, and extracellular enzymes [9].

The treatment of infectious diseases have become now an immediate requirement in the current scenario due to the emergence of multi-drug resistant bugs which are prone to cause Nosocomial and Opportunistic infections. In 2014, the CDC estimated around 75,000 deaths in U.S. due to Hospital Acquired Infections (HAI) wherein 50% of the cases occured outside the ICU [10]. It has been studied that the cause of emerging drug resistance is due to the misuse of antibiotics which leads to genetic mutations in the pathogens. During the early 1960's, betalactamase stable cephalosporins and penicillins were enough to treat most of the Staphylococcal or Enterobactericiae infections. But in the present scenario, there are hardly any drugs to treat multi drug resistant bugs. The effect of natural pigments against multidrug resistant organisms like MRSA, ESBL E. coli, Multi Drug Resistant (MDR) Mycobacterium tuberculosis, Salmonella enterica, etc have been studied. Studies show that a sponge-associated fungi isolated from Indonesia showed a good activity against multi drug resistant Salmonella enterica with an inhibition zone of 14.72 mm [11]. The secondary metabolites extracted from Aspergillus spp., and Penicillium spp exhibited a very good antibacterial activity against MRSA, ESBL E. coli, Klebsiella, and Pseudomonas [12]. There are not many reports on the antimicrobial studies on Multi Drug Resistant Organisms (MDRO) with fungal extracts.

II. MATERIALS AND METHODS

A. Procurement Of The Fungus And Media Used

Chlamydosporum (Strain No. 3020) was procured from the National Fungal Culture Collection Institute (NFCCI), Pune, India. The culture was maintained in Potato Dextrose Agar (PDA). The optimised media for the pigment production contained 2% Glucose, 0.2% each of peptone and beef extract, 0.05% of Magnesium Sulphate as the trace element, 0.05% and 0.1% of Potassium di-hydrogen phosphate and Di-Potassium hydrogen phosphate respectively at the pH of 6.0 and temperature at 29±2°C. 1.5-2% of Agar was added as the solidifying agent.

B. Extraction and Quantification of the intracellular pigment

1) *Extraction of the Intracellular pigment:* The fungus produced intracellular pigment and unlike the extracellular pigments, these do not diffuse into the media. Therefore, the pigment was extracted from the fungal mycelia as per the protocol laid in [13]. The fungal mycelia was transferred aseptically from the optimised media using a sterile cork borer into Potato Dextrose Broth (PDB) under submerged fermentation conditions for 10 days at 29±2°C. The fungal mat was then separated by centifugation at 8000 rpm. The extraction of the pigment was done using ice cold chloroform by homogenizing the mycelia with sterile sand (HIMEDIA, AR Grade) several times until the mycelia became colourless. The extracts collected were filtered using Whatmann No. 1 filter paper, and then evaporated to dryness using a rotary vacuum evaporator.
2) *Estimation of biomass and Pigment Quantification:* Following incubation, the mycelia was separated from the broth by centrifugation for 15 min at 8000 rpm and filtration using Whatmann No. 1 filter paper. The mycelial mass was thoroughly washed with de-ionised water thrice until the water became colourless. The washed mycelia was then dried overnight in a hot air oven at 70°C till the mycelial weight was constant. The dry weight was expressed as g/L [14]. The concentration of the pigment was quantified by measuring the absorbance of the crude pigment extracts at 530 nm in a UV-Visible spectrophotometer (Schimadzu UV-1700, pharmaspec). The units were expressed as units of absorbance ml^{-1} of the broth [13].

C. Purification of the pigment

1) *Thin Layer Chromatography (TLC):* Analytical TLC was performed on TLC plate 20 cm × 20 cm (GF 254 60; Merck 250 mm thick). The chloroform extract of the crude pigment of *F. chlamydosporum* was spotted aseptically with a capillary tube on the TLC plate according to the standard procedure. The spot was air dried and eluted with the mobile phases Chloroform:Methanol:Acetic acid (60:35:5) following which the bands were visualised under visible light and UV light (short wavelength and long wavelength). The Rf value of the compound was determined using the standard formula [15]. After drying, the pigment spot was scraped, mixed with chloroform, and centrifuged at 4000 rpm for 20 min. The supernatent was kept in a vial for evaporation and the dried extracts were collected for the bioassay activities.

D. Antimicrobial assay

1) *Screening of the pigment extracts against the Nosocomial drug resistant pathogens:* The antimicrobial assay with the partially purified extract of *F. chlamydosporum* against the Nosocomial pathogens was evaluated by the Kirby Bauer method. Six pathogens namely *Staphylococcus aureus* ATCC®1708™ (MRSA), *Escherichia coli* ATCC®2326™(ESBL), *Pseudomonas aeruginosa* ATCC®27853™, *Acinetobacter baumanii* ATCC®19606™, *Serratia marcescens* ATCC®14756™ and *Candida albicans* ATCC®10231™ procured from the American Type Culture Collection (ATCC) were used for the study. The Kirby Bauer assay was performed as outlined in the NCCLS document M2-A9 [16]. The pathogens were grown in Nutrient Broth for 24 hrs at 37±2°Cand the turbidity was set to 0.5 McFarland standards. The pigments were dissolved in DMSO at 5 mg/ml concentration. The disc diffusion assay was performed on Mueller Hinton Agar. The inoculum set to 0.5 McFarland standards was evenly spread onto the Mueller Hinton Agar plates, allowed to dry and sterile discs of 6 mm incorporated with the pigment was placed at equal distance on the plates. DMSO was used as the negative control. Appropriate positive controls as given in the NCCLS standard guidelines were used for the assay [17]. The plates were then incubated for 18 - 24 hrs at 37±2°Cexcept for the MRSA plate which was incubated at 35±2°C to overcome hetero resistance.
2) *Minimum Inhibitory Concentration (MIC) determination of the pigment extracts:* MIC of the pigments exhibiting inhibitory activity was determined by well diffusion assay. Different concentrations of the pigments ranging from 5000 µg/ml to 9.7 µg/ml were prepared by dissolving the extract in 10% DMSO. The inoculum suspension of the test pathogens in Nutrient broth were adjusted to 0.5 McFarland according to the standard procedures and was evenly swabbed onto Mueller Hinton Agar plates under aseptic conditions. The different concentrations of the pigment were incorporated onto sterile discs and placed at equidistance from one another. DMSO was used as the negative control. The concentration of the positive controls was 100 µg/ml and were as follows: Linezolid for MRSA, Piperacillin tazobactam for ESBL *E. coli*, Gentamicin for *A. baumanii*, Chloramphenicol for *S. marcescens*, and Itraconazole for *C. albicans*. The plates were further incubated at 37°C and were observed for the zone of inhibition after 18 - 24 hours. The procedure for agar diffusion method was performed as laid in the NCCLS document, M7-A7 [18].

III. RESULTS

A. Growth of the fungus on Optimised media

The organism exhibited orange red pigment on the optimised media and grew radially all over the media with irregular margins. The reverse side of the plate showed intense pigmentation with reddish orange colour. The maximum pigment production on the optimised media covering complete petridish was seen on the 12th day of incubation. The colony morphology of the fungus is given in Figure 1.

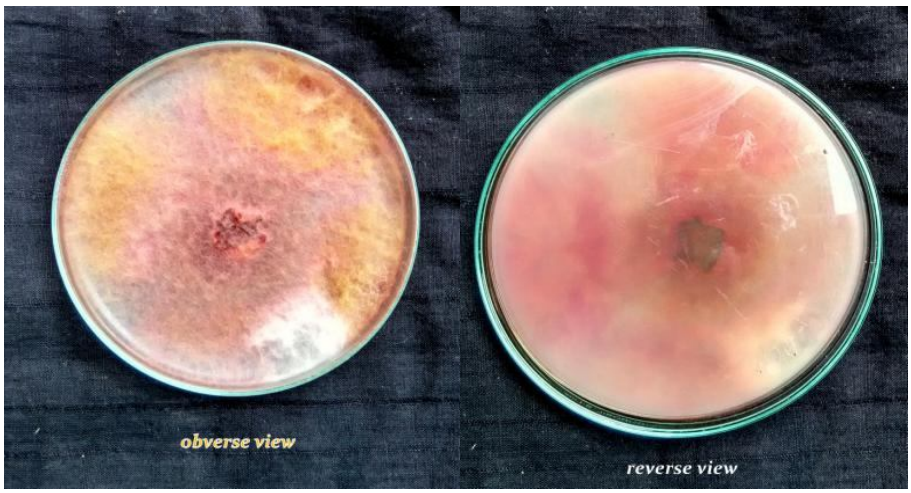

Fig. 1. Reverse and obverse view of colony morphology of F. *chlamydosporum*

B. Effect of Incubation time on pigment and biomass production of F. chlamydosporum

Influence of incubation time with F. chlamydosporum was studied for 17 days starting from day 1 of the inoculation. F. chlamydosporum exhibited higher pigment production on the 12th day, whereas the mycelial biomass continued to increase till the 15th day, after which there was no significant increase (Figure 2).

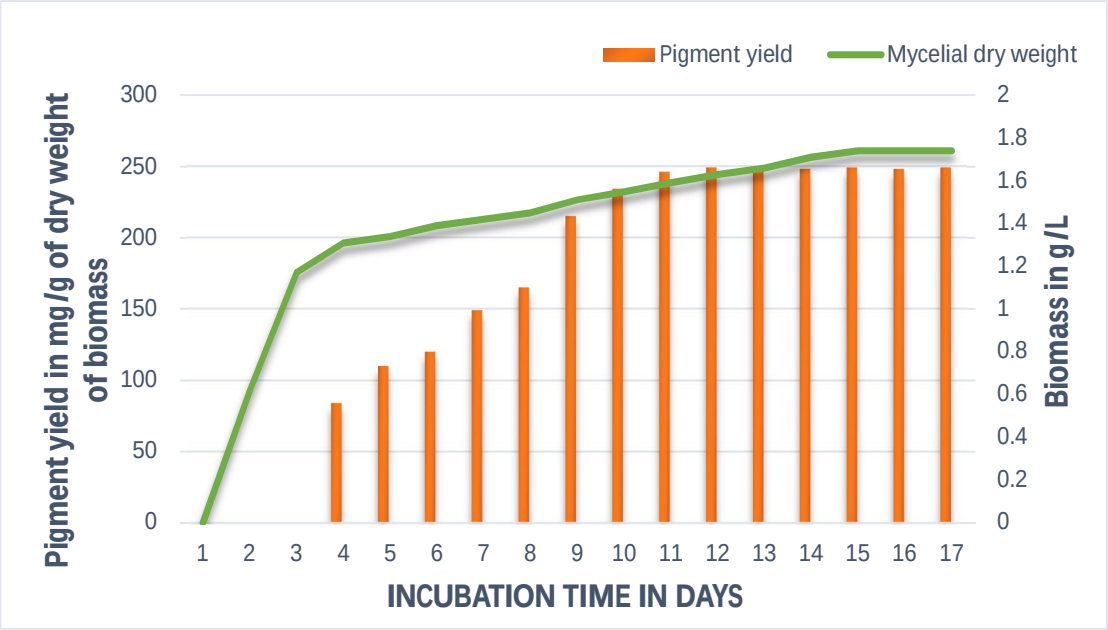

Fig. 2. Effect of incubation time on pigment and biomass production of *F. chlamydosporum*

C. Purification of the pigments

1) *Thin Layer Chromatography (TLC):* The pigments were separated on TLC and the solvent system for the mobile phase was standardised for better separation of the pigment compounds. The chloroform extracts of the pigment was separated on the TLC plate with standardised mobile phases (Chloroform:Methanol:Acetic acid {60:35:5}The pigment was separated into a single band with Rf= 0.864 cm.
2) *Preparative TLC:* The compound fraction of the pigment with high purity was obtained by scraping off the respective band of the Rf value= 0.864 cm; dissolving it in chloroform and separating it from the silica gel by filtration. The compound was then subjected to a rotary evaporator for drying.

D. Antimicrobial assay

1) *Screening of the pigment for antimicrobial activity and MIC determination:* Out of the six test organisms, MRSA, ESBL *E. coli*, *A. baumanii*, *S. marcescens*, and *C. albicans* were inhibited by *F. chlamydosporum* pigment (Table 1). Further, MIC determination of the pigment was evaluated by Kirby Bauer method which is depicted in the Figure3. The MIC of the pigment against MRSA, ESBL *E. coli*, *A. baumanii*, *S. marcescens*, and *C. albicans* were found to be 39.06, 625, 312.5, 39.06 and 312.5 µg/ml respectively.

Table 1. Screening for antimicrobial activity of pigment by *F. chlamydosporum*

Sl. No.	Test pathogens	Pigment from *F. chlamydosporum*
1	Methicillin Resistant *Staphylococcus aureus* (MRSA)	+
2	Extended Spectrum BetaLactamase *Escherichia coli* (ESBL *E. coli*)	+
3	*Acinetobacter baumanii*	+
4	*Pseudomonas aeruginosa*	-
5	*Serratia marcescens*	+
6	*Candida albicans*	+

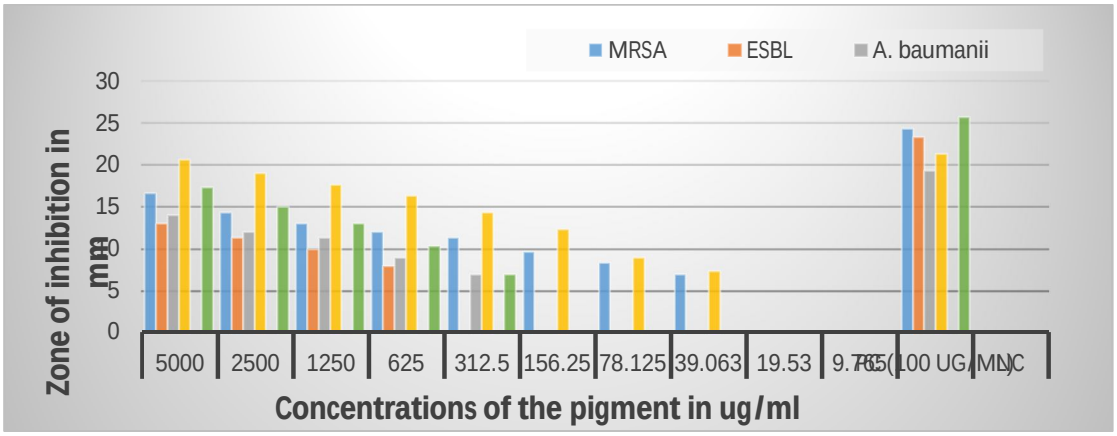

Fig. 3. Minimum inhibitory concentration (mic) of the pigment against test organisms

2) *Statistical Analysis:* All the results were expressed as mean ± SD (n=3). Experimental data were analysed by Mutivariate ANOVA using the SPSS software (SPSS Inc.) 20.0. version depending on the nature of data set. Means were separated by post hoc analysis – Tukey HSD with the level of significance at $P<0.05$.

IV. CONCLUSION

The present study concludes that the pigment extracted from *F. chlamydosporum* has potential application in therapeutic researches and pharmaceutical field. The pigment was able to inhibit the emerging drug resistant pathogens at a minimum concentration when compared with the current antibiotics. This study has generated information in terms of extraction procedures of the pigment, purification techniques, and antimicrobial assay against the test organisms. Thus the optimisation and further study of natural pigments prove to be an emerging research area in the field of microbiology and pharmacology.

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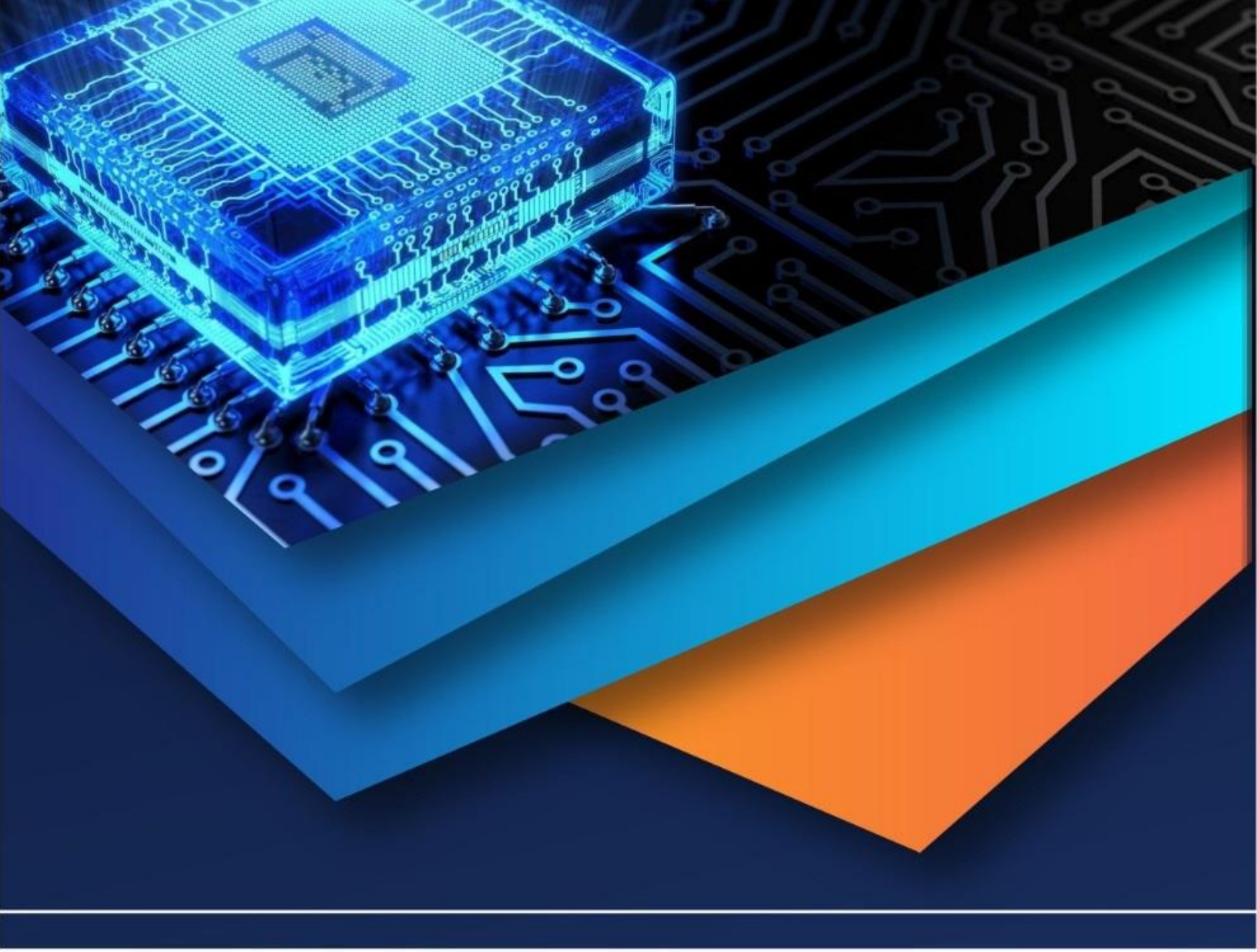

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