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Antimicrobial Effects of Medicinal Plants on Pathogenic Food Bacteria

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Abstract: ***Food Pathogens were isolated from different street food samples and molecular identification of the isolates revealed the presence of Escherichia species, Pseudomonas aeruginosa, Klebsiella pneumonia and staphylococcus aureus. All the bacterial strains were tested against crude extracts of Justicia adhatoda and plectranthus amboinicus for their antimicrobial properties. Plectranthus amboinicus showed a higher and significant antimicrobial property, however Justicia adhatoda showed less activity when used in lower concentrations.***
Keywords: Food Pathogens, Antimicrobial

I. INTRODUCTION

Disease and Infections caused by microorganisms that contaminate the food supply are a frequent reminder of the complex food web that can pose a potential health hazard. The consequences are most severe in the vulnerable populations of the very young, the elderly, and those with compromised immune systems. Though many pathogens can contaminate food, some pathogens like the *norovirus* and *Salmonella typhi* are harboured in human reservoirs and contaminate the food through the excreta of infected individuals (3). Food borne illness are inflammations or infections of the gastrointestinal tract caused by food or beverages that are either contaminated or harbour the harmful bacteria, viruses and parasites. The gastro intestinal tract is a series of long connected organs starting from mouth to the anus. Most foodborne illnesses are acute and most people can recover without any esteemed medical treatments. However rarely foodborne illnesses may lead to more serious complications and preventing such illness becomes difficult as the standard treatments can also have a significant side effects or ineffective in preventing recurrent infections by making the pathogens resistant to the drugs (16).There are various opportunistic pathogens that can cause serious threat in immunocompromised individuals and leads to more severe complications, such pathogens can cause acute and chronic illnesses even induce cancer, and damage vital organs such as the liver kidney and brain by producing aflatoxins or mycotoxins (18) Plants are the naturally available resource, which has been the foundation for traditional systems of medicine for thousands of years providing mankind with new remedies and continue to provide new beneficial therapeutic properties. Plants are used for their useful properties such as Anti-Fungal, Anti-viral, anti-bacterial, anti-cancer Anti-Tumour, Anti-fungal, Anti-oxidants, anti-malarial Etc which are being exploited for developing new drug targets and pharmacological actions against wide range of Ailments (20) *Justicia adhatoda* commonly known as adhatoda in Tamil, adhatoda, vasaka or Malabar nut is a native plant to Asia widely used in traditional medicinal systems The whole of the plant has been extensively used as a medicinal plant due to the phytochemicals such as alkaloids, tannins, saponins and the most important vasicine, a quinazoline alkaloid. The leaves, Stem, roots and flowers of adhatoda plant have been extensively used in medicinal systems for more than thousands of years to treat respiratory disorders such as asthma, old cough and helps in clearing bronchiole disorders. It is very useful in preventing bleeding through nose, mouth and also in stools, adding to that it has been used to control both internal and external bleeding as it has anti-inflammatory properties and antibacterial properties. These plants are dried and used for treating malaria, and the juice from the plant juice has been used for treating dysentery, diarrhoea etc.

The vasicine yield has been measured to be around 1% by dry weight (9) *Plectranthus amboinicus* is an herb commonly known as Omavalli in Tamil language, it's a medicinally important herb as the whole plant has been extensively used for its medicinal property as it contains Anthocyanins, Steroids, Terpenoids, Fatty acids, Coumarins, Tannins, Saponins and other important alkaloids (10) The leaves of Amboinicus has been strongly used for the treatment of coughs, sore throats and nasal problems. Apart from Treating other infections and health complications. The plant can be cultivated throughout the year for medicinal preparations which can be used for treating malarial fever, Renal and vesical calculi, Chronic asthma and bronchitis (11). The isolated colonies were sequenced to identify the presence of various food pathogens and further tested against two medicinally important plants for their antimicrobial properties and to develop a Lead drug compound which can be beneficial against the developing resistance of the microorganisms.

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II. MATERIALS AND METHODS

1) *Media:* Different culture medias were employed for the isolation and culturing of the bacterial isolates. Nutrient agar was used as base to culture all microorganisms from the samples. Selective and differential media such as mac-conkey agar, Eosin methylene blue, pseudomonas isolation agar, mannitol salt agar, sabourad dextrose agar and salmonella shigella agar obtained from Hi-media laboratories were used. The media was prepared in distilled water and autoclaved at 121°C for 15 minutes.
2) *Collection of Sample:* 6 samples from various localities of Chennai were selected and sugar cane samples were obtained in sterile containers and plastic covers. Medicinal plants were obtained from Kaivalya, Ayurveda nursery in Chennai.
3) *Isolation of Bacterial strains:* Isolation was done by standard serial dilution methods. Colony forming units were recorded and each colony was further isolated to obtain pure culture. The bacterial isolates were streaked on to different selective and differential media and was incubated at 37°C for 24-48 hours. Standard Biochemical tests were employed to identify the genus of the bacterial isolates.
4) *Molecular Characterization:* DNA was isolated from the 4 different bacterial isolates which showed different colony characteristics. DNA was amplified through polymerase chain reaction and obtained PCR products were sent for 16SrRNA sequencing, which is a signature sequence specific to each and every organism in species level. Sequencing was done by using the 8 forward and 1490 reverse primers. Sequencing of the isolates were outsourced from Bio-Kart Private limited.
5) *Crude Extract Preparation:* Plants were cleaned and shade dried till it becomes powder. Shade Dried plants were mechanically grinded and the dry weight was measured. Ethanol and distilled water extracts were prepared in 1:10 volume with powdered leaves. Fresh leaves were weighed and crushed to obtain fresh crude extract, the extracts were stored in air tight containers.
6) *Phytochemical Screening:* Plant extracts were screened for their phytoconstituents by various standard phytochemical tests.
7) *Fourier Transform Infra-Red:* Powdered Plant extracts were dried and sent for FTIR analysis to detect various Phyto functional groups present in the leaves. The Spectroscopy data was compared with spectroscopy databases.
8) *Thin Layer Chromatography:* In Thin layer chromatography a solid and a liquid phase is used to separate the compounds based on the affinity with the two phases. Liquid Phases were prepared by using Chloroform and methanol in the ratio 9:1, Ethyl acetate and hexane in the ratio of 1:1 and allowed for saturation in the chromatogram chamber.
9) *Antimicrobial Activity:* Antimicrobial activity was carried out by Kirby Bauer method, well diffusion and disc diffusion methods were used. Muller Hinton Agar was used for the assay. Disc diffusion method for antimicrobial assay was carried out using Fresh Leaf Aqueous extracts of *Plectranthus amboinicus* and *Justicia adhatoda* 100µl was inoculated on to the sterile discs, impregnated on to the agar plates. The sterile discs were carefully loaded with the plant extracts and allowed to dry before impregnating on to the agar plates with the isolated bacterial culture. Standard antibiotic discs were also used to test out the efficacy of the Bacterial isolates. The clear zone of inhibition was recorded.

III. RESULTS

1) *Isolation of Bacterial Strain:* The colony forming units were counted by using an electronic colony counter, CFU were too low and too numerous to count between dilutions 10^{-3} and 10^{-6} respectively. 8 different bacterial isolates were obtained from different sugar cane samples by standard serial dilution methods. Out of 8 isolates, 3 had similar colony morphology and 5 were selected for further characterization of the isolates.
2) *Strain Characterization:* One isolate was able to grow on sabourad dextrose agar and inferred as yeast species. EMB agar showed Green metallic sheen colour which indicates the presence of fecal coliforms, Pseudomonas isolation agar showed pigmentation which is very specific to *Pseudomonas species*, Mac-Conkey agar showed characteristics lactose fermenting colonies which indicated the presence of *Klebsiella species* along with *E. Coli Species.* Mannitol salt agar showed presence of *staphylococcus species* showing fermentation of the mannitol. Standard biochemical characterization also confirmed the presence of *Klebsiella species, Staphyloccocus Species, Escherichia species* and *Pseudomonas species.*
3) *Molecular Characterization:* Molecular characterization of the 4 isolates were done by using 16SrRNA sequencing, the sequencing results were analysed by using BLAST tool from NCBI and the results showed *Klebsiella pneumonia, Staphylococcus aureus* and *Pseudomonas aeruginosa.* (Table 1)
4) *Phytochemical Screening:* Phytochemical analysis revealed that *Justicia adhatoda* and *Plectranthus amboinicus* contained Alkaloids, Tannins, saponins, Terpenoids, flavonoids, phenolic compounds in fresh aqueous and ethanolic extracts (Table 2)
5) *FTIR Analysis:* Based on the functional group vibrations observed when exposing the compounds to infra-red spectra, the peaks are recorded and later compared with available infra-red database. The Dried extracts of *Plectranthus amboinicus* and *Justicia adhatoda* shows the presence of multiple functional groups which indicates the presence of Phyto Functional groups in leaves

of these plants. Carboxylic acids, Phenols, Amides, Alcohol group, Aromatic compounds, Alkenes functional groups were identified using FTIR analysis. These Show the presence of phytochemicals carrying hydrogen bond -OH functional group which is an integral part for most of the phenolic compounds such as flavonoids and tannins and also the OH bend of the carboxylic acids reveals the presence of saponins and glycosides.

Table 1: 16srRNA Sequencing of The Isolates

PCR ASSAY	TARGET GENE	PRIMER NAME	PRIMER SEQUENCE	PRODUCT SIZE
Klebsiella pneumonia	16srRNA	8-Forward 1490-Reverse	AGAGTTTGATCCT GACTTACCAGGGT	764BP
Staphylococcus aureus	16srRNA	8-Forward 1490-Reverse	AGAGTTTGATCCT GACTTACCAGGGT	769BP
Pseudomonas aeruginosa	16srRNA	8-Forward 1490-Reverse	AGAGTTTGATCCT GACTTACCAGGGT	777BP

Table 2: Phytochemical screening

PHYTOCHEMICAL TESTS	Justicia Adhatoda		Plectranthus amboinicus	
	Fresh Extract	Ethanol Extract	Fresh Extract	Ethanol Extract
Alkaloids	+	-	+	+
Tannins	+	-	+	+
Saponins	+	-	+	-
Terpenoids	+	+	+	+
Phenolic compounds	+	+	+	+
Flavonoids	+	+	+	+
Proteins	+	-	+	-
Glycosides	+	-	+	-

6) *Antimicrobial Activity Of The Extracts:* *Klebsiella pneumonia* has showed Clear zone of inhibition for *Justicia adhatoda* distilled water extract (18mm ,21mm) and *Plectranthus amboinicus* (16mm). *Staphylococcus aureus* has showed Zone of inhibition for *Justicia adhatoda* (17mm) and *Plectranthus amboinicus* (22mm), *Escherichia species* has showed zone of inhibition for *Justicia adhatoda* (14mm) and *Plectranthus amboinicus* (15mm), *Pseudomonas aeruginosa* has showed clear zone of inhibition for *Justicia adhatoda* (14mm) and *Plectranthus amboinicus* (16mm). The Control showed no activity against the Isolates (Table 3). Comparison of the two plant extracts where plectranthus amboinicus showing significantly higher activity (figure 1)

7) *Antibiotic Sensitivity:* Klebsiella pneumonia was sensitive to Chloramphenicol, Gentamycin and Streptomycin, showed resistance against ampicillin and penicillin. Staphylococcus aureus was sensitivity to Gentamycin, Streptomycin and chloramphenicol and intermediate zones to Ampicillin and penicillin. Escherichia species was sensitive against gentamycin and streptomycin and showed resistant to ampicillin, chloramphenicol and penicillin. Pseudomonas aeruginosa was sensitive to gentamycin, streptomycin and chloramphenicol and was resistant against ampicillin and penicillin. (Table 4)

Table 3: Efficiency of Plant Extracts on Isolated food pathogens

Microbial Isolates	Control (mm)	Justicia adhatoda Distilled water 50(µl) (mm)	Justicia adhatoda Fresh Leaf (100µl) (mm)	Plectranthus Ambonicus Fresh Leaf(100µl) (mm)
Klebsiella pneumonia	0	18	21	16
Staphylococcus aureus	0	0	17	22
Escherichia species	0	0	14	15
Pseudomonas aeruginosa	0	0	14	16

Table 4:Antibiotic Sensitivity of Isolated Food Pathogens

Bacterial isolates	Ampicillin (mm)	Gentamycin (mm)	Streptomycin (mm)	Chloramphenicol (mm)	Penicillin (mm)
Klebsiella pneumonia	0	10	9	18	0
Staphylococcus aureus	0	18	17	19	0
Escherichia species	0	17	19	0	0
Pseudomonas aeruginosa	0	18	19	13	0

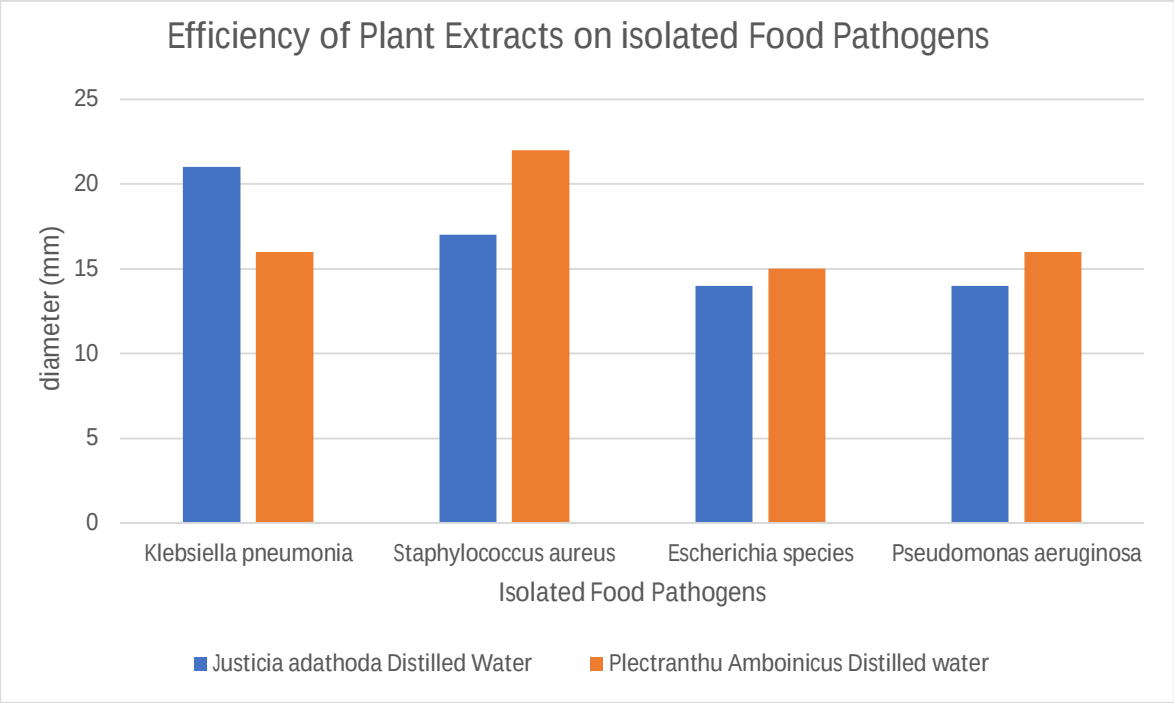
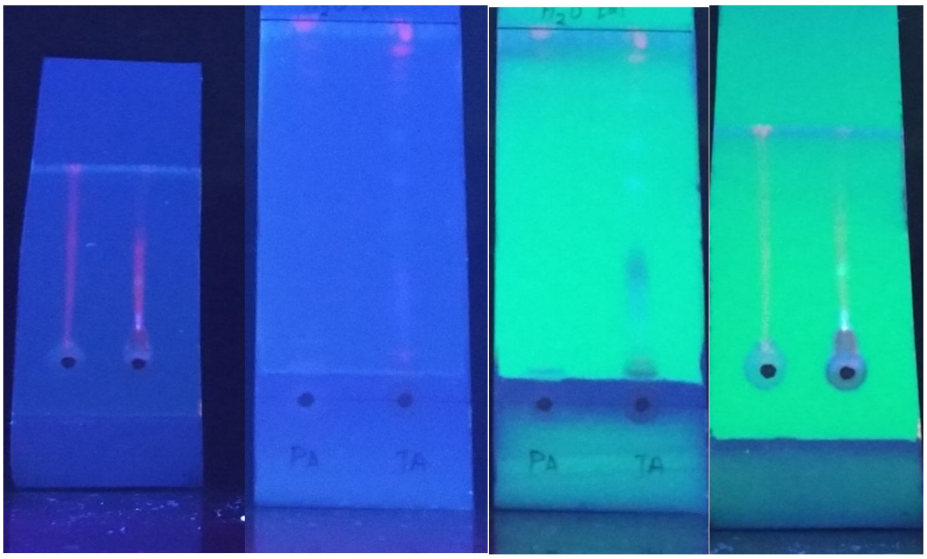


Fig 1: Efficiency of plant extracts on Bacterial Isolates

8) *Thin Layer Chromatography:* Thin Layer chromatography was performed as a preliminary study to assess the suitable solvent for extraction of various bioactive compounds. A drop of the Extracts was allowed to elute in the TLC chamber and the spots were measured using a standard Scale and observed under U-V light at 302 and 365nm. The Retention factor values of the extracts with different solvent systems are recorded with standard scale, the spots were measure with the solvent front and measure using the Equation of retention factor. Clear bands were visualized for *Plectranthus amboinicus* and very faint bands were observed for *Justicia adhatoda*. Rf values of *Plectranthus amboinicus* were ranging between 0.3 to 0.9 whereas for *Justicia adhatoda* it was between 0.4 to 0.9. The bands indicate the presence of various phytocompounds that can be further purified by using appropriate solvent system to extract specific bioactive compounds. (Table 5 and figure 2)



Bands of J.A and P.A at 365 nm and 302 nm in solvent 1 and 2

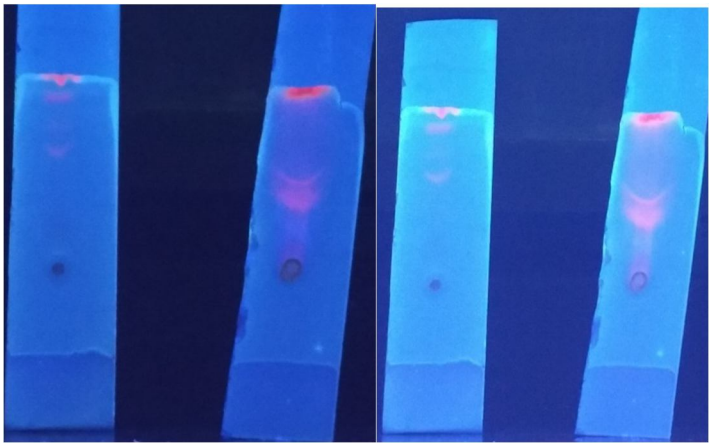


Figure 2 : Thin layer chromatography

Table 5: Rf Values of the extracts using TLC

Sl. No	Extract Name	Solvent System 1		Solvent system 2	
		No. Of Spots detected	Rf Value	No. of Spots detected	Rf Value
1	*Justicia adhatoda* Fresh Extract	2	0.66 0.9	3	0.4 0.53 0.88
2	*Plectranthus amboinicus* Fresh Extract	2	0.78 0.9	2	0.3 0.52

IV. DISCUSSION

Natural products have been the single most effective source of leads for the development of drugs from various compounds derived from leads of plant origin have been used to cure a number of disease and health conditions (14) Isolation of Enteric pathogens revealed that the food samples were contaminated with faecal contamination and also possible sewage contamination. Majority of the samples have been contaminated with *Escherichia coli, Staphylococcus aureus, klebsiella species* and *pseudomonas species* as a cause of defective personal hygiene along with bacteria found in environment and on handling of the food. The sugar cane samples sold in the street are without any proper hygiene or handling. The usage of ice, the water being used, the utensils all play a major

role in contamination of the samples. Dirty cloths, unhygienic environment, utensils lead to higher possibility of bacterial contamination. Food borne illness occurs by the transmission of these pathogens via food to humans by consumption of these contaminated foods (21) Most of the juices were served in re-used utensils by washing in bare hands leading to contamination of *staphylococcus species* also it might harbour preformed toxins of *staphylococcus aureus* which is a known source of food poisoning. Inadequate washing and absence of good hygienic conditions also contribute to coliform and *Escherichia coli* contamination. Occurrence of *Pseudomonas species* might be due to sewage mixing or due to unhygienic surroundings (19) All the shops are in open environment and are always susceptible to atmospheric air, vehicular transmissions, dust and flies leading to more contamination of the food that are being sold in the streets.

Molecular characterization was done using 16srRNA sequence which sequences the signature region which is specific only to the particular organisms and amplifies the sequences which can be later compared with the available databases to confirm the species (8)

Justicia adhatoda and Plectranthus amboinicus has been considered as medicinal plants due to their Phytoconstituents which have been used to treat various disorders and diseases by traditional methods, these plants can be used as a source of lead compound against various pathogens as they constitute various bioactive compounds which can be attributed towards synthesizing New drugs to treat diseases .The Leaves of the plants were crushed and filtered to estimate the efficacy of the plants when used as a whole and to test the phytochemicals present when used as a crude. Standard extraction methods were not employed as these methods have already been standardized, very few other extraction methods such as infusion and decoction have been less employed (17). Traditional herbal preparations involve the use of Crushing the Plant and using the raw extract as source of drug, however to detect the particular compound of interest it is essential to use Standardized methods to isolate such compound and further create a drug compound. Phytochemical screening has showed the presence of various bioactive compounds and phytochemicals present in these plants such as glycosides, alkaloids, terpenoids, phenolic compounds which has been reported to be effective antimicrobial substances (2). In addition to being effective against bacteria these compounds also exhibit various inhibitory effects against various other disorders and ailments. Flavonoids are the well-established naturally available antioxidants and other class of complex compounds are alkaloid which are useful against pathogens and other infections as they possess antimicrobial properties (4). These naturally occurring phytocompounds are effective in various ways and can be used in multiple ways as drug targets for various complications. In any Biological groups, its functional groups influence the biological activity as they contribute in various ways such as their crystal structure their solubility, stereochemistry etc. These properties in turn influence various other factors and functional group analysis plays a very important role in understanding the physiochemical properties of the extract (15). FTIR analysis of the Leaves has showed the presence of various phytochemicals with OH bonds as functional groups indicating that hydroxyl groups are integral part of the phenolic phytocompounds such as flavonoids tannins etc (13) (12) Which in turn has various antioxidant properties and anti-inflammatory activities apart from phenolic compounds (5), it also indicates the presence of various other organic compounds such as amino acids, chlorophyll, lignin's carbohydrates might also be present.

The plant extracts were inoculated in to the agar diffusion wells containing the isolated organisms, showed no activity against the pathogens these negative results could be due to the difference in the phytochemicals present or the composition of the extracts and the methodology of the antimicrobial tests (6). The plant as crude extract did not diffuse properly into the agar wells thereby inhibiting its antimicrobial action. The plant extracts were later tested on Sterile Discs, allowed to dry before inoculating on to the agar well platers. After incubation for 24hours the plant extract discs showed effect against the isolated organisms. The zone of inhibition was measure and compared with other standard antibiotics to test out the efficacy of the plant extracts. Justicia adhatoda showed significant activity when used in higher concentrations when used with distilled water extract, Plectranthus amboinicus contains water content and showed higher activity against the pathogens when tested in crude form, Plectranthus amboinicus seemed to be a promising plant for the use of developing an active lead compound.

TLC is a very easy and efficient method to separate and study the various compounds present by subjecting them to the polarity of the compounds, based on their affinity these compounds tend to separate based on their polarity and these bands can be visualized effectively by various spraying agents and observed directly under U- V light in different Wavelength. Ethyl acetate: Hexane and chloroform: Methanol Solvent systems showed clear separation of bands as these solvent systems have a polar and nonpolar properties that can be very well utilized for the separation of various phytochemicals based on their polarity (1). Hexane and chloroform are non-polar solvents whereas Ethyl acetate and Methanol are polar solvents. Various phytochemicals provide different Rf values and this provides a very important aspect in understanding the use of appropriate solvent systems and their polarity in various ratios which will help in separation of compounds from the extracts. This can be achieved by analysing the Rf values of the compounds in different solvent systems which reflects an idea about their polarity (7)

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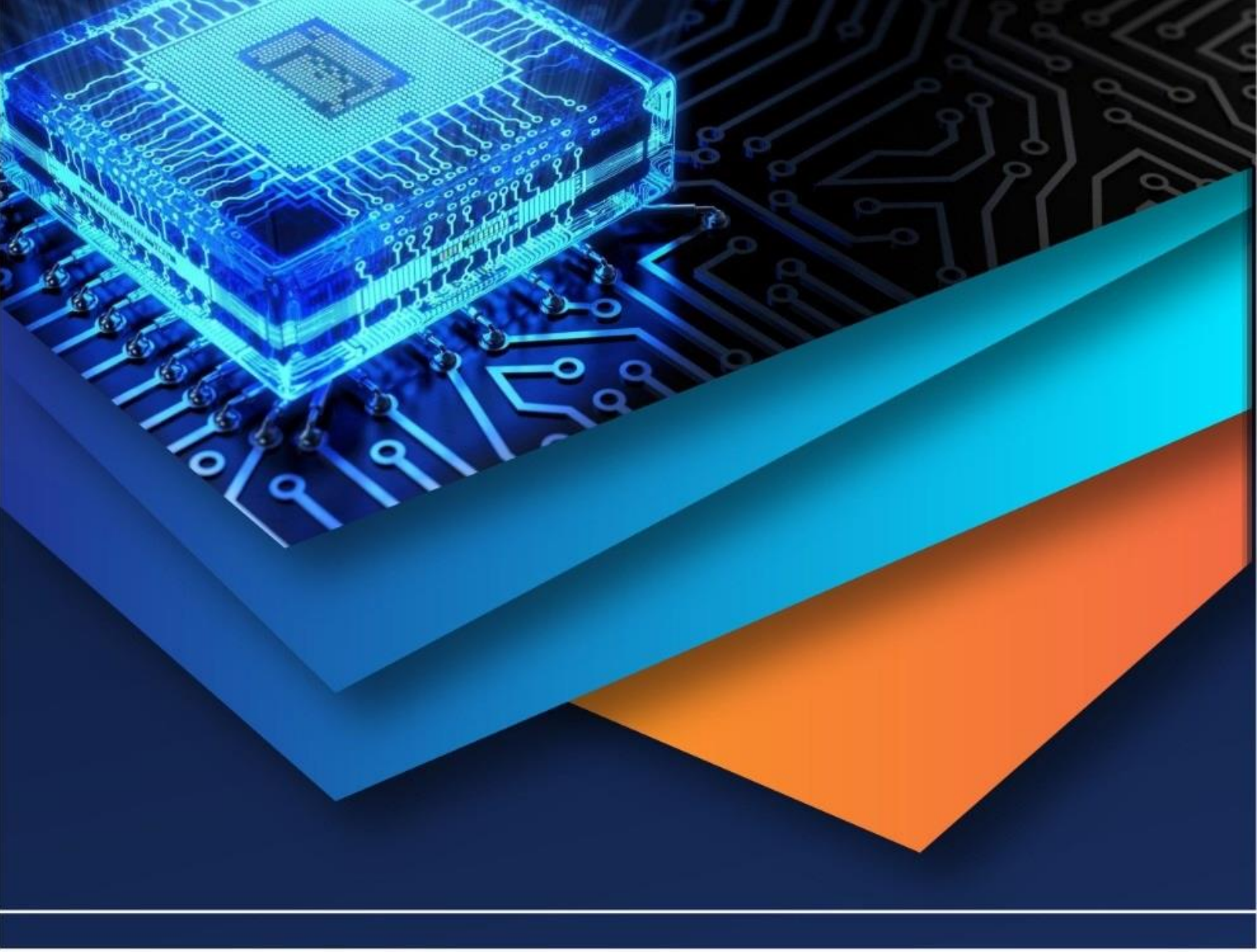

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