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Demonstration of Anti-Inflammatory and Metabolic Stability Assay of Andrographis Paniculata

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Abstract: ***In vitro drug metabolism studies, which are inexpensive and readily carried out, serve as an adequate screening mechanism to characterize drug metabolites. Liver S9 fractions include Phase I and II metabolic enzymes, are relatively inexpensive, easy to use. Cytochrome P450 enzymes are responsible for metabolizing many endogenous and xenobiotic molecules encountered by the human body. It has been estimated that 75% of all drugs are metabolized by cytochrome P450 enzymes. The human liver microsomal protein is one of the most important obstacles for designing personalized medicines. Liver microsomes are widely used to determine In vitro intrinsic clearance (CL_{in}), which can then be scaled to predict In vivo hepatic clearance. Microsomes can also be used to elucidate metabolic routes. We demonstrated that anti-inflammatory and metabolic stability assay by using Andrographis paniculata. The human PBMC were isolated from the healthy donar. The TNF-alpha were analyzed by human PBMC. This article demonstrate that the percentage of inhibition of inflammation were identified (Graph pad prism 3.0) as 2.832 for rolipram and 9.935 for extract.***

Keywords*: *Invitro* drug metabolism, Cytochrome P450 enzymes, Liver microsome, Human PBMCs *Andrographis paniculata

I. INTRODUCTION

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants and is a protective response involving immune cells, blood vessels and molecular mediators. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation, such as mononuclear cells. Inflammation is mediated by a variety of soluble factors, including a group of secreted polypeptides known as cytokines. Inflammatory cytokines can be divided into two groups: those involved in acute inflammation and those responsible for chronic inflammation. Tumor necrosis factor-alpha (TNF-alpha) has been shown to play a key role in these processes and thus might be a potential therapeutic target. TNF promotes the inflammatory response, which, in turn, causes many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and refractory asthma. These disorders are sometimes treated by using a TNF inhibitor.

The metabolic stability assays provide a means to measure the rate of disappearance of a test compound over time in either microsomal or hepatocyte incubations, and these data are used to calculate intrinsic clearance. Microsomal assays primarily assess metabolism by the cytochrome P450 system (phase I enzymes) while hepatocyte assays more broadly assess the overall cellular metabolism of the test compound (phase I and phase II enzyme pathways). We provide metabolic stability assays for all small molecule formulations such as pharmaceuticals, industrial chemicals and consumer products. Liver metabolism is the major route for elimination for many drugs in humans and animals. The native ability of hepatic enzymes to metabolize a drug is commonly referred to as “intrinsic clearance” and is used to determine overall hepatic clearance, which takes into account additional factors such as hepatic blood flow and drug/protein binding. The liver is the most important site of drug metabolism in the body. Approximately 60 % of marketed compounds are cleared by hepatic CYP-mediated metabolism. Liver microsomes are subcellular fractions which contain membrane bound drug metabolising enzymes. Microsomes can be used to determine the *in vitro* intrinsic clearance of a compound. The use of species-specific microsomes can be used to enable an understanding of interspecies differences in drug metabolism. Subcellular fractions such as liver microsomes are useful *in vitro* models of hepatic clearance as they contain many of the drug metabolising enzymes found in the liver. They are easily adaptable to high throughput screens which enable large numbers of compounds to be screened rapidly and inexpensively. The most prominent group of drug metabolizing enzymes is the super family of cytochrome P450s.

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Andrographis paniculata is an annual herbaceous plant in the family Acanthaceae, native to India and Sri Lanka. It is widely cultivated in Southern and Southeastern Asia, where it has been traditionally used to treat infections and some diseases. Mostly the leaves and roots were used for medicinal purposes. The plant is known in north-eastern India as Maha-tikta, literally "king of bitters" and known by various vernacular names. It is also known as Nila-Vembu in Tamil.

II. MATERIALS AND METHODS

A. *Preparation of human PBMCs from blood*

Dilute the cells with 2-4x the volume of buffer. Carefully layer 35 ml of diluted cell suspension over 15 ml of Ficoll-paque in a 50 ml conical tube. Centrifuge at 400xg for 30-40 minutes at 20°C in a swinging bucket rotar without brake. Aspirate the upper layer leaving the mononuclear cell layer to a new 50 ml conical tube. Fill the conical tube with buffer, mix, and centrifuge at 300xg for 10 minutes at 20°C. Carefully removed supernatant completely. For the removal of platelets, resuspend the cell pellet in a 50 ml of buffer and centrifuge at 200xg for 10-15 minutes at 20°C. Carefully remove the supernatant completely. Repeat above step. Most of the platelets will remain in the supernatant upon centrifugation at 200xg. Resuspend cell pellet in an appropriate amount of buffer and proceed to magnetic labeling.

B. *Preparation of Microsomes From Rat Liver*

The overnight fasting animals were sacrificed by cervical dislocation. Liver was removed rapidly and placed in the ice cold homogenizing buffer in an ice bath. Then the liver was perfused by inserting 10 ml syringes with 22-G needle into the liver vasculature and the buffer was injected till the effluent was cleared and colorless (~10 ml buffer for rat liver). Excess moisture was removed by blotting on paper towels and the tissue was weighed. 3 ml of ice cold homogenizing buffer was added and the liver was minced into small pieces with surgical scissors. Then the liver was divided into ~4 g each. Further break apart the tissue on ice with a held blender for 3-5 sec. The tissue was homogenized on ice using a motor driven teflon pestle with 10 strokes. Then the Homogenate was transferred into an ice cold labeled 1.5 ml centrifuge tubes. Homogenate was centrifuged for 15 min at 12,500×g, 4□C. Supernatant was decaned carefully into a 1.5 ml ultracentrifuge tube and discard the pellet. The supernatant was ultracentrifuge for 70 mins at 105,000 ×g, 4□C. Then the supernatant was decaned and the peelet was resuspended in 8 ml icecold pyrophosphate buffer. Then the pellet was homogenized using the hand-held blunder for 3 to 5 sec. Then the tubes were rebalanced and ultra centrifuged for 45 mins at 105,000 ×g, 4□C. The supernatant was decaned the pellet was resuspended by adding 6 ml ice cold microsome buffer with hand held blunder. Further the pellet was homogenized with potter-type Teflon pestle and transferred into a clean tube. Teflon pestle was washed with 2 ml ice cold microsome buffer and combined.

C. *Phytochemical Analysis*

1) *Test for carbohydrates:* To 200 µl of plant extract, 100 µl of Molisch's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.
2) *Test for tannins:* To 100 µl of plant extract, 200 µl of 5% ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins.
3) *Test for saponins:* To 200 µl of plant extract, 200 µl of distilled water was added and shaken in a graduated cylinder for 15minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.
4) *Test for flavonoids:* To 200 µl of plant extract, 100 µl of 2N sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.
5) *Test for alkaloids:* To 200 µl of plant extract, 200 µl of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.
6) *Test for quinines:* To 100 µl of extract, 100 µl of concentrated sulphuric acid was added. Formation of red color indicates presence of quinones.
7) *Test for glycosides:* To 200 µl of plant extract, 300 µl of choloroform and 10% ammonia solution was added. Formation of pink color indicates presence of glycosides.
8) *Test for cardiac glycosides:* To 50 µl of extract, 200 µl of glacial acetic acid and few drops of 5% ferric chloride were added. This was under layered with 100 µl of concentrated sulphuric acid. Formation of brown ring at the interface indicates presence of cardiac glycosides.
9) *Test for terpenoids:* To 50 µl of extract, 200 µl of chloroform was added and concentrated sulphuric acid was added carefully. Formation of red brown color at the interface indicates presence of terpenoids.

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10) *Test for triterpenoids:* To 150 µl of extract, 100 µl of chloroform shaken with concentrated sulphuric acid was added. Lower layer turns yellow on standby indicates the presence of triterpenoids.
11) *Test for phenols:* To 100 µl of the extract, 200 µl of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols.
12) *Test for coumarins:* To 100 µl of extract, 100 µl of 10% NaOH was added. Formation of yellow color indicates presence of coumarins.
13) *Steroids and phytosteroids:* To 100 µl of plant extract equal volume of chloroform is added and subjected with few drops of concentrated sulphuric acid appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicates the presence of phytosteroids.
14) *Phlobatannins:* To 100 µl of plant extract few drops of 2% HCL was added appearance of red color precipitate indicates the presence of phlobatannins.
15) *Anthraquinones;* To 100 µl of plant extract few drops of 10% ammonia solution was added, appearance pink color precipitate indicates the presence of anthraquinones.

D. Metabolic Stability Assay

50 µl of liver microsomes was added into the tubes containing 445 µl of trisHcl buffer. This mixture was vortexed for 5 seconds. 5 µl of 1mM Embelin was added and vortexed well. 75 µl of assay mixture was immediately removed and added to the tubes containing 75 µl of ice cold acetonitrile solution (0 min as control tube). Assay mixture was incubated in water bath at 37^0C for 60 minutes. After 5 minutes 75µl of the assay mixture was taken from the water bath and immediately added into the tube containing 75 µl of acetonitrile solution. This procedure was repeated for 15 min, 30 min, 45 min and 60 min respectively. Tubes were centrifuged at 14,000 g for 10 minutes. Supernatant was collected and protein concentrations were determined by using standard methods.

E. TNF- α inhibition in human PBMCs

1) Cell treatment: The stock solution of extracts and pure compound were prepared in DMSO and then diluted to the desired concentration in which the final maximum concentration of DMSO in the media was not more than 0.1% DMSO. Cells were seeded at a density of 106 cells/ml in 96well microtiter plate. Cells were pretreated with different extracts at 20 g/ml and then stimulated with LPS. Positive controls were treated only with LPS where as in negative control wells medium containing 0.1% DMSO was added. Cells were maintained at 37°C in a humidified incubator under an atmosphere supplemented with 5% CO_2.

2) Quantitation of cytokines: Supernatants were collected for cytokine analysis. Cytokine levels were quantitated using enzyme-linked immunosorbant assay (ELISA) kit from R and D systems, according to the manufacturer's instructions. Supernatant (100 µl) was added to antibody-coated polystyrene wells and incubated for 2 h. After washing, the plates were incubated with biotin-labeled anti-cytokine antibody for 2 h. The plates were washed and incubated for 20 min with a streptavidin/horseradish peroxidase conjugate. The plates were washed and incubated with trimethylbenzidine (TMB) and peroxide, to detect the horse radish peroxide. The reaction was stopped by the addition of 2N sulphuric acid and the absorbance read at 540 nm and 450 nm on a titertek Multiskan MCC/340 microplate reader.

F. Analysis of whole blood TNF-alpha assay

Steps	Reagent	Blank	Std/Sample
1. Add reagents	Std/sample	-	100 µl
	Conjugate	-	100 µl
2. Incubation	Cover plate and incubate overnight at 4°C		
3. Wash	Wash all wells five times		
4. Add Reagents	Ellman's	200 µl	200 µl
5. Incubation	Cover plate and incubate 30-90 minutes RT with gentle shaking		
6. Read	Read plate at a wavelength between 405-420 nm		

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III. RESULTS AND DISCUSSION

The liver microsomes are subcellular fraction derived from the endoplasmic reticulum of hepatic cells. The microsome preparation consist of pooled microsome isolated from female IGS Sprague dawley rat livers. We were analysed metabolic stability on liver microsome by using Andrographis Paniculata. It is a ayurvedic medicinal plant used to analyse the drug activities. The presence of phytochemical components were analyzed shown in fig. 1. We also used human PBMC to estimate the protein data with the help of BSA. It is a standard method used for estimation of protein were shown in fig. 2. We used metabolic stability assay protocol to analysed the drug activities. The different time periods were involved this method (5 times point) an extensively used high through out assay in the pharmaceutical industry. We reached same conclusion regarding stability using only 2 time points (t=0 & 60 min). The result of this analysis were shown in fig. 3. Microsomes have a reddish brown colour, due to presence of the heme. Because of the need for a multi part protein system. Microsomes are necessary to analyse the metabolic activity of CYPs. These are highly abundant in liver of rats, mice humans. Researchers use microsomes to mimic the activity of the ER in a test tube conduct experimentally that's require protein synthesis on a membrane. There are two kind of metabolism generally involved in drug metabolism. However the metabolic profiles obtained with liver microsomes may not be a accurate representation of the metabolism encountered in hepatocytes or invivo because, as S9 liver fraction account for phase I metabolism and phase II metabolism where as liver microsome account only for phase I metabolism. Next we analyzed the normal human whole blood assay analysis. The bioavailability of drugs were analyzed with the help of this analysis. The level of TNF-α were analysed by different concentration (0.1,0.3,1,3,10,30,100 mg/ml) by using human PBMC were shown in fig. 4. IC_{50} determination the log dose of extract / standard drug was plotted vs. the percentage of inhibition and regression analysis of log dose–response curve was used to calculate IC_{50} by using Graph Pad Prism 3.0 (Graph Pad Software). The percentage of inhibition were identified based on the TNF-alpha activity as shown in TABLE I. The IC50 identified as 2.832 for rolipram and 9.935 for extract. The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. The values are typically expressed as molar concentration. It is commonly used as a measure of antagonist drug potency in pharmacological research. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition in vitro. It is comparable to an EC_{50} for agonist drugs. EC_{50} also represents the plasma concentration required for obtaining 50% of a maximum effect in vivo.

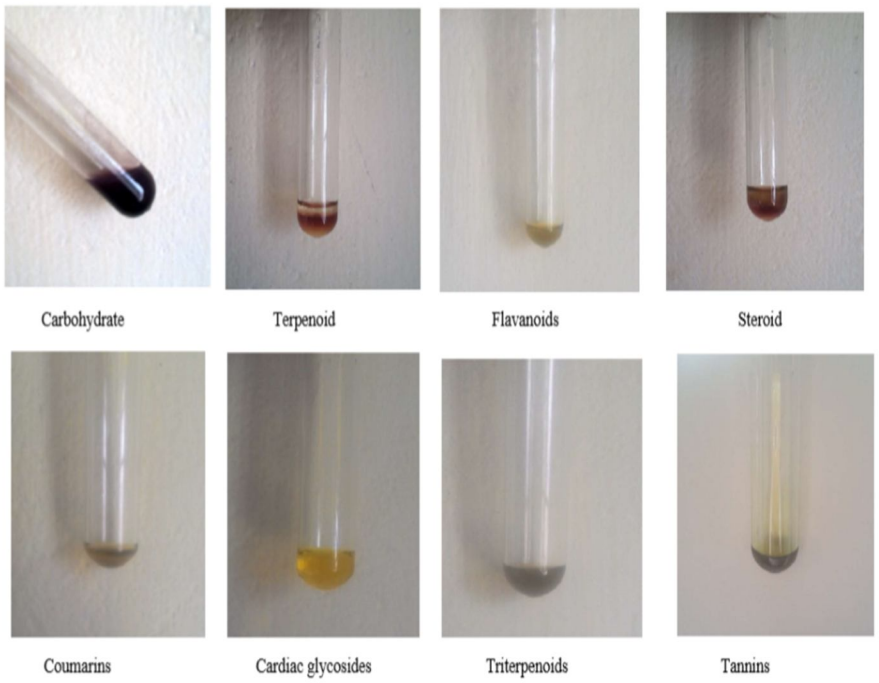


Fig. 1 Phytochemical analysis

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Concentration (mg/ml)	OD at 540nm		Mean OD	Sample OD	
				#DIV/0!	#DIV/0!
1	0.150	0.136	0.143	0.115	0.474
0.5	0.100	0.148	0.124	0.097	0.158
0.25	0.107	0.104	0.106	0.09	0.035
0.125	0.092	0.096	0.094	0.09	0.035
0.0625	0.099	0.078	0.089	0.079	-0.158

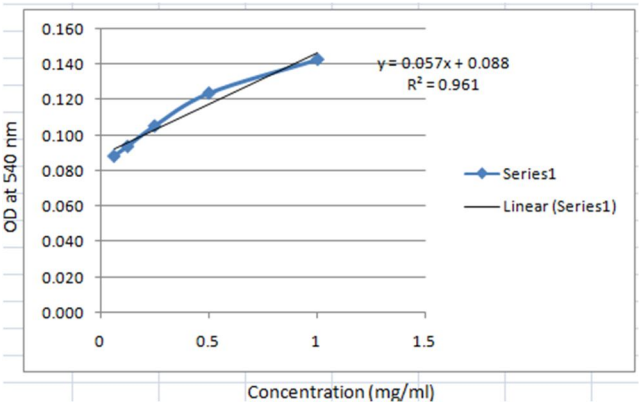
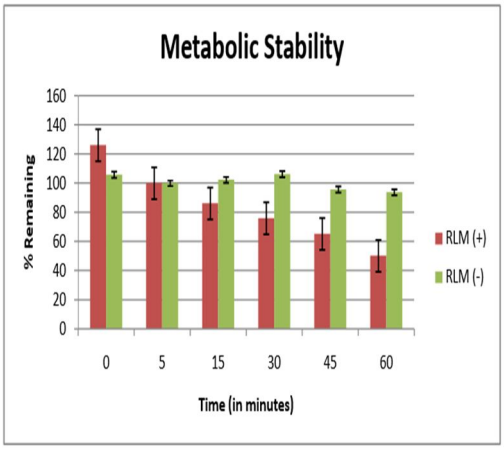


Fig. 2 Estimation of protein-BSA

Embelin INVITRO STUDY		
Rat Liver Microsomes		
Sample ID	**Area**	**% Remaining**
RLM I 0 (+)	176552	126
RLM I 5 (+)	140090	100
RLM I 15 (+)	120650	86
RLM I 30 (+)	106370	76
RLM I 45 (+)	91370	65
RLM I 60 (+)	70340	50
RLM I 0 (-)	191440	106
RLM I 5 (-)	180940	100
RLM I 15 (-)	185210	102
RLM I 30 (-)	192040	106
RLM I 45 (-)	173020	96
RLM I 60 (-)	169590	94

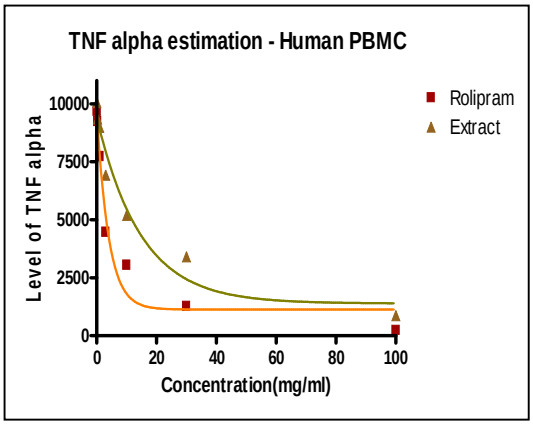
Extract Stock-1	618130



Minutes	0	5	15	30	45	60
RLM (+)	126	100	86	76	65	50
RLM (-)	106	100	102	106	96	94

Fig. 3 Metabolic stability

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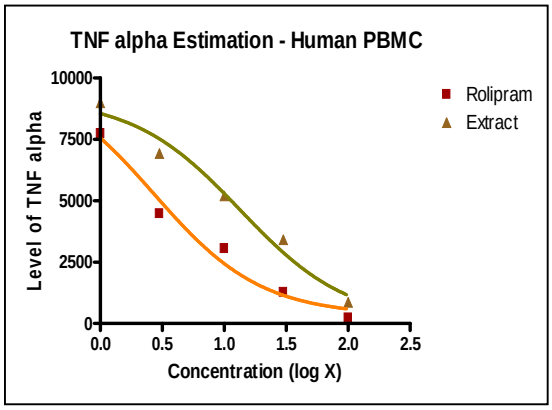


Fig. 4 TNF alpha Estimation – Human PBMCs

TABLE I The percentage of inhibition – IC50

	Rolipram	Extract
Sigmoidal dose-response		
Best-fit values		
BOTTOM	300.9	454.8
TOP	10090	9750
LOGEC50	0.4521	0.9972
EC50	2.832	9.935
Std. Error		
BOTTOM	393.3	721
TOP	412.3	375.7
LOGEC50	0.09875	0.136
95% Confidence Intervals		
BOTTOM	-790.7 to 1393	-1547 to 2456
TOP	8945 to 11235	8706 to 10793
LOGEC50	0.1780 to 0.7262	0.6197 to 1.375
EC50	1.506 to 5.324	4.166 to 23.69
Goodness of Fit		
Degrees of Freedom	4	4
R^2	0.9901	0.9823
Absolute Sum of Squares	869337	1.24E+06
Sy.x	466.2	556.8
Data		
Number of X values	7	7
Number of Y replicates	1	1
Total number of values	7	7
Number of missing values	0	0

IV. CONCLUSION

Historically liver microsomes and hepatocytes have been employed as metabolic stability screens but these have their limitations. Microsomes have a limited enzymatic make-up whereas hepatocytes are cost-prohibitive, labor extensive. Indeed, the *in vitro* and *in*

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vivo CYP induction studies are helpful in interpreting initial animal pharmacokinetic studies. Metabolic pathways are divided into phase I and phase II reactions, and both phases often occur in parallel for a particular compound. In phase I reactions, enzymes modify the parent drugs via hydrolysis, oxidation and reduction, increasing the polarity and also the excretion of the drug. Most often, phase I reactions are preparative stages for further reactions exposing reactive sites to the molecule structure for the subsequent phase II processes (i.e., conjugation reactions). Phase II reactions are often considered "true" detoxifying reactions, as the conjugation of bulky and polar groups most often terminates the activity of the substrate and enhances elimination. Glucuronidation is the main phase II reaction in all mammals, with the other important pathways being sulfation, methylation, acetylation and conjugation with amino acids or glutathione. Our analysis demonstrates that metabolic activity on *Andrographis paniculata* is much more efficient and cost effective manner and also IC50/EC50 determined how much a particular drug or other substances is needed to inhibit a biological process.

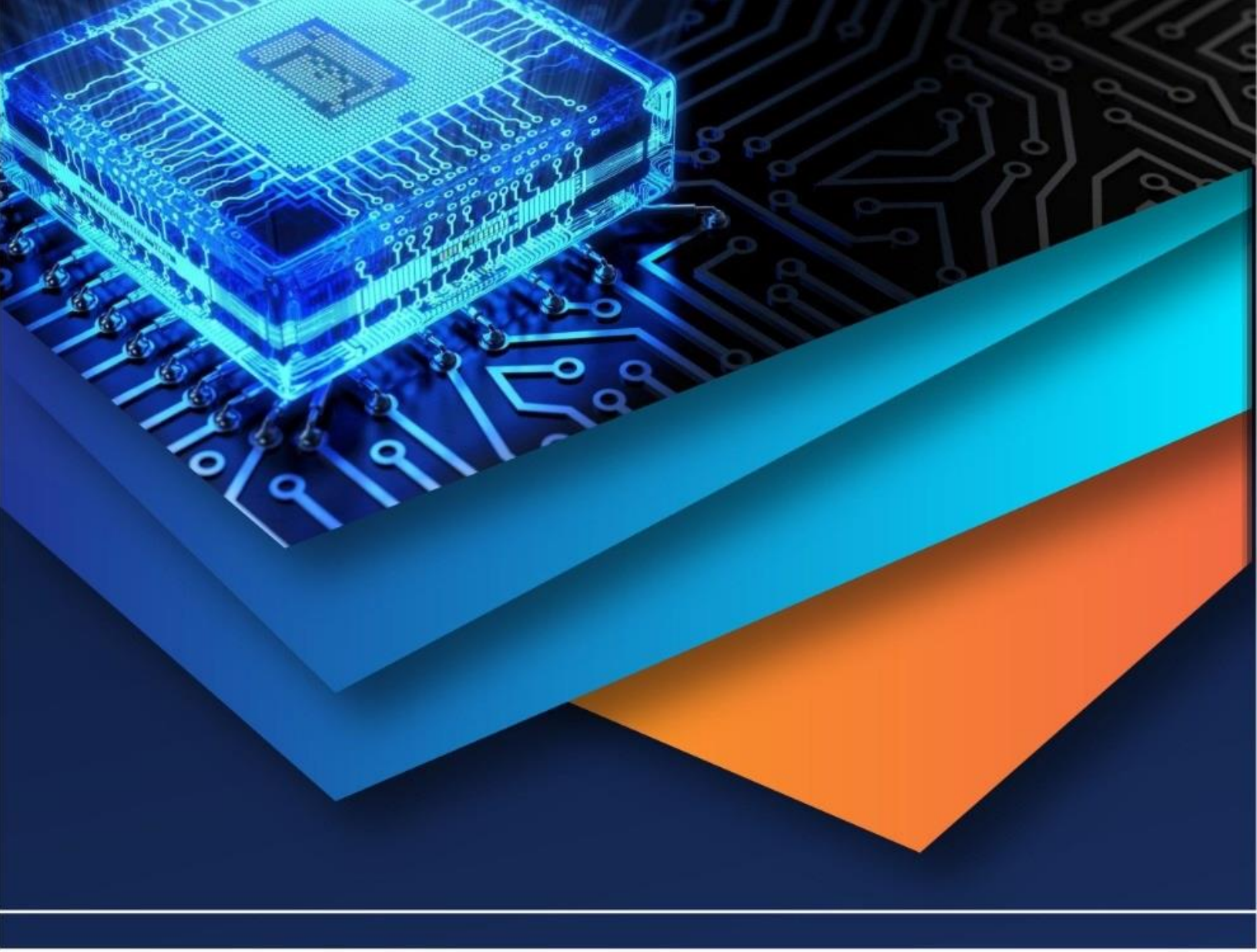
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