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BIOLOGICAL ACTIVITY OF VALACYCLOVIR DERIVATIVES OF [(2-AMINO-9-((2-HYDROXY ETHOXY)ETHYL)-1H-PURINE-6(9H)-ONE)].

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Abstract

Valacyclovir is a synthetic purine nucleoside analogue that has been approved by FDA as an antiviral drug. One of the most commonly used valacyclovir which works at lower concentration than acyclovir is a very old drug that was used during Severe Acute Respiratory Syndrome Coronavirus 2 infection worldwide. DPPH radical scavenging assay, Hydroxyl scavenging, DNA binding and protecting assay, antimicrobial activity and ATPase activity of 2-amino-9-((2-hydroxy ethoxy) methyl)-1H-purin-6(9H)-one derivatives were assessed. Among the selected guanine derivatives SA-A to J, SA-D analogue possessed comparatively highest DPPH radical scavenging potential, hydroxyl radical scavenging activity. The guanine derivatives B, C, F, H comparatively showed excellent DNA protecting activity. SA-A to SA-J responded differentially to gram positive and gram negative bacterial cultures. H+/K+ ATPase inhibition activity of tested guanine analogues revealed that SA-E has higher inhibitory potency. In the present investigation, the biological screening of 2-amino-9-((2-hydroxy ethoxy) methyl)-1H-purin-6(9H)-one derivatives responded differentially and to summarize SA-D analogue is a strong antioxidant and SA-E is comparatively potent Gastric H^+ /K^+ ATPase inhibitor.

Key words: 2-amino-9-((2-hydroxy ethoxy) methyl)-1H-purin-6(9H)-one, valcyclovir, Antioxidant assay, Antimicrobial assay.

INTRODUCTION

Rapidly mutating pathogenic RNA viruses pose a serious threat to humanity. The COVID-19 pandemic caused by the novel Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) has proved the shortage of universal antiviral agents. Viruses being obligate parasites, its genome encodes the minimum set of enzymes and proteins necessary for the replication and assembly of viral particles. For the biosynthesis of building blocks (monomers), host cell enzymes are used, so the reproduction of a virus is possible only inside the virus infected host cell. According to the literature, the first type of drugs prevents the penetration of the virus into the cell, the second type disrupts the replication of the viral genome inside the cell. Nucleoside analogues which are active antiviral agents, and their mechanism of action rely on the type and position of the substituents. These nucleoside analogues are also used to treat infections caused by bacteria, protozoa and fungi. The huge urge for creation of antiviral drugs is not confined to the consideration of classical approaches, and as new technologies are emerging, new approaches are also being developed. The emergence of drug resistance in pathogenic microorganisms is a serious problem for humanity. Guanine and related nucleobases are the promising molecular tools for designing functional supramolecular assemblies because of their unique topological pluralism. Recent Spectacular results of various chemical fields, from supramolecular chemistry to chemical biology, from soft matter to catalysis. In this paper, to gain accurate information and to demonstrate that multidisciplinary investigations shed a bright light on the diversity that guanines, synthetic guanines and related nucleobases are potent drugs to combat the infection. In natural products and synthetic medicines, nitrogen heterocycles are highly important structural motifs responsible for various biological activities. This has quenched the interest of medicinal chemists towards the synthesis of various compounds having nitrogen heterocycles as useful medicines to treat various diseases and it is evident by the survey that a large number of pharmaceutical products are nitrogen heterocycles[1].

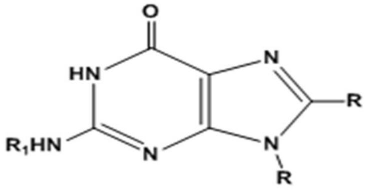


Figure 1: General structure of Guanine.

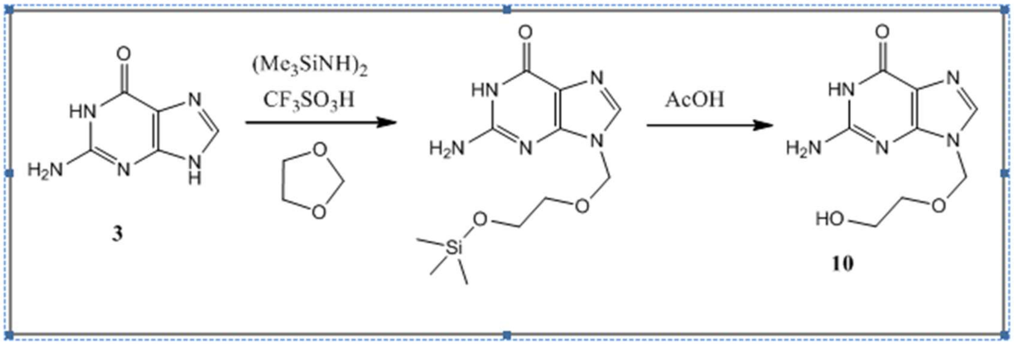
The most prominent member 9-(2-Hydroxyethoxymethyl) guanine also known as acyclovir has been used to have many synthetic modifications to the guanine structure. This compound together with different side chain analogues has long been known to possess antiviral activity [2].

Valacyclovir and its several analogues are of current interest due to their antiviral activity. Valacyclovir, is L-valine 2 [(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl) methoxy]ethyl ester having antiviral activity and their derivative synthesis and assessing their biological activity is gaining more interest[3]. Valacyclovir could be converted to valcyclovir upon oral administration which is inhibitory against Herpes simplex virus-1(HSV-1), Herpes simplex virus-2(HSV-2), and Vericella zoster virus. An effective antiviral drug valacyclovir, compared to acyclovir, acts at low dosing and achieves higher blood plasma level. Acyclovir which is an acyclic nucleoside analogue of guanosine was discovered in the year 1974 by Gertrude B. Elion and her group. They published the research article entitled, “the selectivity of action of an antiherpetic agent, 9-(2-Hydroxyethoxymethyl) guanine” which was the first finding that described the specific treatment for the viral infections and was later known as acyclovir [4,5,6,7,8,9,10].

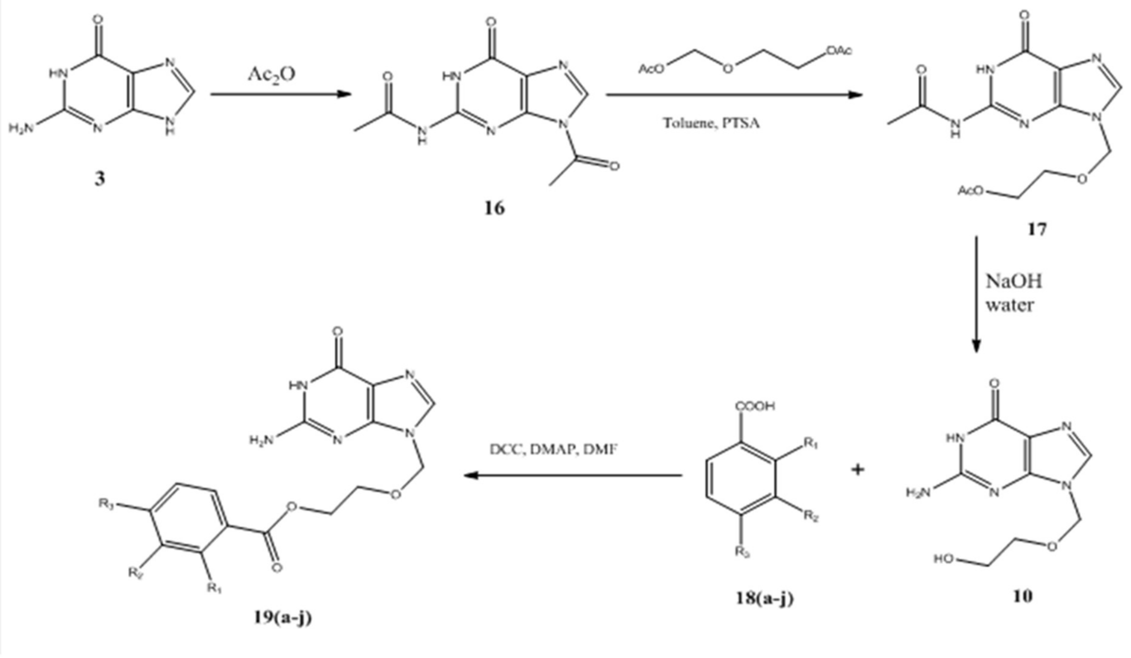
MATERIALS AND METHODS

MATERIALS

2-amino-9-((2-hydroxy ethoxy) methyl)-1H-purin-6(9H)-one derivatives, DMF, nutrient broth, agar, BHT, DPPH, methanol, EDTA, ascorbic acid, FaCl3, Deoxyribose, H2O2, CT-DNA, Fenton's reagent, bromophenol blue dye, agarose, ethidium bromide, TAE buffer, gel documentation, Omeprazole, Kreb's ringer buffer, 2mM HEPES-Tris, Sucrose-EDTA buffer (122mM NaCl, 4.8mM KCl, 25.2mM NaHCO3), ANSA, Ammonium molybdate. All other chemicals were purchased from Sisco research laboratory, Mumbai, India. All reagents were of analytical grade.



Scheme 1: Synthesis of acyclovir using guanine.



Scheme 2: Synthesis of guanine derivatives 19(a-j).

Compounds	R_1	R_2	R_3
SA-A	H	H	H
SA-B	H	H	NH_2
SA-C	H	H	Cl
SA-D	F	H	H
SA-E	OCH_3	H	H
SA-F	H	H	OH
SA-G	Cl	Cl	H
SA-H	H	H	CH_3
SA-I	H	OH	H
SA-J	H	Cl	H

Biological assay

1. Antioxidant assay.

1a. DPPH radical scavenging assay

DPPH radical scavenging activity was carried out [11]. Briefly, 1 ml of DPPH solution (dissolved in methanol) was mixed with different concentrations of test samples (SA A-J). After vigorous shaking, the mixture was allowed to stand for 20 min at room temperature. Absorbance was measured at 517 nm with UV-VIS spectrophotometer (HITACHI, U-2900) with Butylated hydroxyl toluene (BHT) as a positive control. The radical scavenging activity was measured as decrease in the absorbance of DPPH and calculated and the activity was expressed as EC50 (µg/ml), the concentration required to cause 50% DPPH inhibition.

1b. Hydroxyl radical scavenging

The reaction mixture containing different aliquots of compound sample 0-300µg, deoxyribose (10mM), H_2O_2 (10mM). $FeCl_3$ (5mM0, EDTA (1mM) and ascorbic acid (5mM) in potassium phosphate buffer (50mM, pH 7.4) was allowed to stand for 60 min at 37^{O}C [12]. The reaction was terminated by adding TCA (5% w/v) in a boiling water bath for 15 min. The absorbance was measured at 535 nm against the blank. The inhibition of the oxidation of deoxyribose was calculated against the control using UV-VIS spectrophotometer. BHT was used as positive control and radical scavenging potential was expressed as EC_{50} value.

2. DNA binding and protecting assay.

A series of analogues (SA A-J) were synthesized from the guanine derivatives. The complexes are found to be soluble in dimethylformamide. Molar conductance values in dimethylformamide indicate the non-electrolytic nature of the complexes. Binding of synthesized analogs with calf thymus DNA (CT- DNA) was studied [13,14]. For the gel electrophoresis experiment, the solution of complexes in DMF (10 mg/ml) was prepared and these test samples (100µg) were added to the Calf Thymus-DNA samples and Fenton's reagent incubated for 30 minutes at 37^{O}C. Agarose gel was prepared in TAE buffer, the gel was carefully stained with EtBr (Ethidium Bromide) solution (10mg/ml) for 10-15 min, the solidified gel attained at approximately 55^{O}C was placed in electrophoresis chamber flooded with TAE buffer. After that 20µl of each of the incubated complex-DNA mixtures (mixed with bromophenol blue dye at 1:1 ratio) was loaded on the gel along with standard DNA marker and electrophoresis was carried out under the TAE buffer system at 100V for 10 min**.** After the gel was visualized under UV light using a Bio-Rad Trans illuminator. The illuminated gel was photographed using a polaroid camera (a red filter and polaroid film were used). The experiments were repeated and results were concordant.

3. Anti-microbial activity.

Antimicrobial activity was determined by disc diffusion method[15], Nutrient broth media was prepared and sterilized at 121^0 C and 151 lbs pressure and different bacterial cultures (*E.coli, S.aureus, S.staphylococcus, Micrococcus, B.subtilis, P.aeruginosa*) and fungal strains (*C.albicans*) were incubated at 37^0 C for 24 hrs, this culture was used for further antimicrobial assays.

Nutrient agar (20 ml) was poured into sterilized petriplates. The plates were allowed to solidify. Bacterial were inoculated separately into respective petriplates by spread plate method. Using Whatmann no 1 required number of discs were formed and placed in each petriplates. The sample compound with different concentrations was loaded on to the discs. The plates were incubated at optimum temperature after 24 hrs and were examined for the presence of antimicrobial inhibition, which was further measured and tabulated.

4. Gastric H^+ /K^+ -ATPase activity

4a. Isolation of parietal cells of stomach:

The fundic stomach portion of sheep soon after sacrificing was collected and it was mixed with Krebs ringer buffer (250mM sucrose, 2mM $Mgcl_2$, 1mMEGTA and 2mM Hepes-Tris of pH 7.4). The upper layer was pinned with the help of needles on the dissection table. Mucosal Scrapings were suspended in 10 volumes of Krebs ringer buffer (pH 7.4) and homogenized with 20 strokes of a mortar driven Telfon pestle Homogenizer. The tissues were discarded and the filtrate was subjected to subcellular fractionation. The pellets so obtained were dissolved in 2ml of sucrose- EGTA buffer and were used as enzyme samples [16].

4b. ATPase activity

ATPase activity was determined [17]. Basal Mg^{2+} dependent ATPase activity was measured in 1.0 ml of the reaction medium consisting of 2 mM/L $MgCl_2$, 2 mM/L ATP and 50mM/l Tris-HCl buffer (pH 7.5), K^+ stimulated and HCO_3^-stimulated ATPase activity was defined as the activity in the presence of 10 mmol / L KCl or 20 mmol / L $NaHCO_3$, minus the activity in the basal medium. The ATPase reaction was started by the addition of the substrate (ATP), carried out at 37˚C for 15 mins and terminated with 1.0 ml ice-cold 20 % TCA, liberated inorganic phosphate from ATP was estimated by Fiske Subbarao's method[18].

(H^+\K^+) ATPase activity in various gastric membranes was determined in 1 ml incubation media, containing 40mM Tris-acetate (pH-7.4), 2mM $MgCl_2$, 2mM ATP, 7mM KCl and 7mM NH_4Cl. The ATPase reaction was started by the addition of the substrate (ATP), carried out at 37°C for 15 minutes and the reaction was stopped.

5. Alpha-amylase inhibition assay

In this study, amylase activity for synthesized compounds was assayed according to Bernfeld method. This assay is based on the oxidation of ketone functional group in synthesized compounds. The principal involved is the test for the presence of free carbonyl group (C=O), so called reducing sugar. One mole of sugar will react with one mole of 3, 5- dinitrosalicylic acid. Simultaneously 3, 5 -dinitrosalicylic acid (DNS) is reduced to 3-amino, 5- nitro salicylic acid under alkaline conditions [9]. The absorbance of the reaction mixture was read at 540 nm using UV spectrophotometer.

RESULTS AND DISCUSSION

1. Antioxidant assay.

1a. DPPH radical scavenging assay

Oxidative damage caused by the reactive oxygen species is reduced by antioxidants. DPPH radical scavenging potential of the analogues is extremely valuable for antioxidant profiles and hence widely applied technique. Among the guanine analogues synthesised, only 7 guanine analogues were assessed for DPPH scavenging activity. The percentage of DPPH activity was comparatively more in guanine derivatives of SA-C, SA-E, SA-G, SA-H, SA-I, SA-J and their EC_{50} values has been tabulated in Table-1 compared to the standard (BHT). SA-D analog possessed comparatively highest activity and SA-H showed comparatively lowest activity.

Table 1: DPPH radical scavenging activity of guanine analogues

SI NO	Guanine Analogues	EC_{50} Value µg/ml
1	BHT	303.71
2	SA-A	No activity
3	SA-B	No activity
4	SA-C	454.83
5	SA-D	438.27
6	SA-E	400.76
7	SA-F	No activity
8	SA-G	415.90
9	SA-H	329.43
10	SA-I	426.60
11	SA-J	417.21

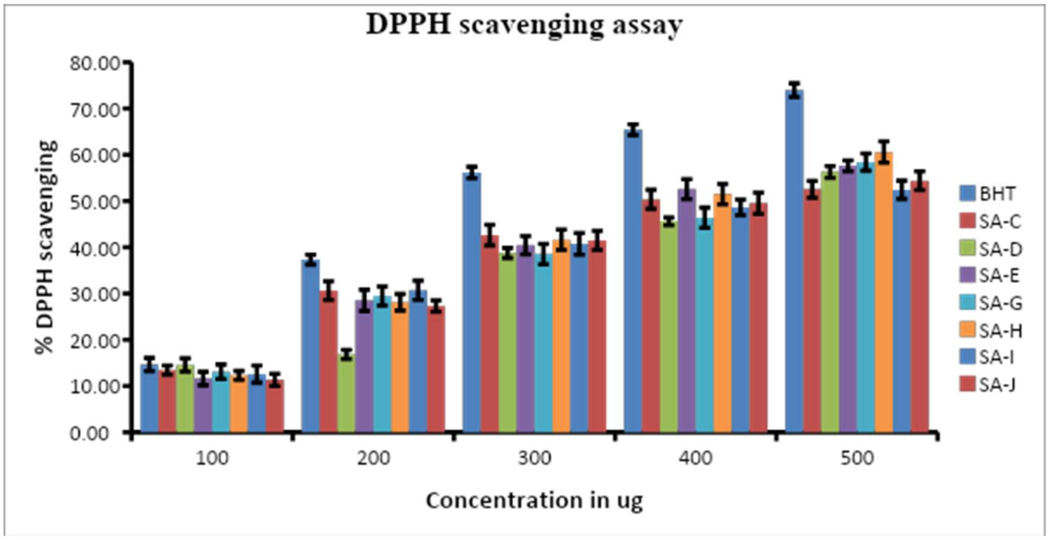


Fig 1: DPPH radical scavenging activity of guanine analogues.

1b. Hydroxyl radical scavenging assay

Only 6 guanine analogues showed hydroxyl scavenging activity. The percentage of hydroxyl activity was more in the presence of guanine derivatives of SA-D, SA-F, SA-G, SA-I, SA-J with EC_{50} values of 272.14, 340.26, 263.81, 327.01, 325.69, 282.18µg/ml respectively; compared to that the standard (BHT) which had an EC50 value of 222.64µg/ml. SA-D showed higher potency and SA-F showed lower potency. The other 4 SA-B, SA-C, SA-E, SA-H, did not show hydroxyl scavenging activity.

Table 2: Hydroxyl radical scavenging activity of Guanine analogues

SI NO	Guanine Analogues	EC 50 Value µg/ml
1	BHT	222.64
2	SA-A	272.14
3	SA-B	No activity
4	SA-C	No activity
5	SA-D	340.26
6	SA-E	No activity
7	SA-F	263.81
8	SA-G	327.01
9	SA-H	No activity

10	SA-I	325.69
11	SA-J	282.18

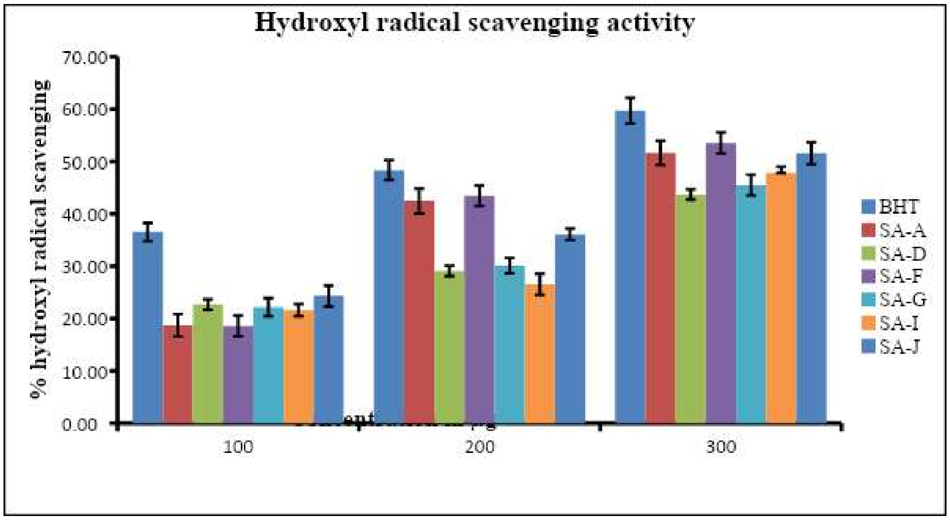


Fig 2: Hydroxyl radical scavenging activity of Guanine analogues.

2. DNA binding and protecting assay.

This assay was used to investigate the ability of the guanine derivatives to protect the CT DNA against damage caused by hydroxyl (OH°) radicals. Hydroxyl free radicals generated by the Fenton's reaction are known to oxidatively induces breaks in DNA strands to yield its open circular, relaxed forms or might be completely degraded. Exposure of CT DNA to Fenton's reagent ultimately results in strand breaks, mainly due to generation of reactive species-hydroxyl radical and subsequent free radical-induced reaction on CT DNA producing base radicals and sugar radicals. The base radicals in turn react with the sugar moiety causing breakage of the sugar phosphate backbone of nucleic acid, resulting in strand break. Addition of CT DNA with Fenton's reagent resulted in degradation of CT DNA. DNA (100µg) was incubated and resulted in the formation of thick DNA bands. The protecting effect on CT DNA is attributed to the presence of guanine derivatives. The protective effect of the guanine derivatives B, C, F, H comparatively showed excellent DNA protecting activity, derivatives such as D, E, G, I moderately protected DNA. The compounds A, J showed comparatively very less activity.

Table 3: Different Components loaded in four wells of Agarose gel electrophoresis

1.	CT DNA+ loading dye
2.	CT DNA + Fenton's reagent
3.	CT DNA + Fenton's reagent +100µg of guanine derivatives

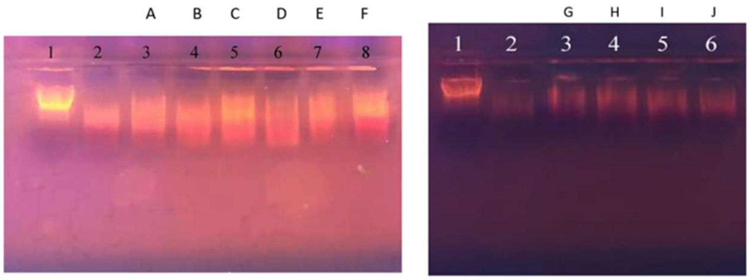


Figure 3: DNA binding studies of Well 1-CT DNA + loading dye, well 2- CT DNA + Fenton's reagent, well 3 to Well 8 were loaded with CT DNA + Fenton's reagent +100µg of guanine derivatives.

3. ANTIMICROBIAL ACTIVITY

In the present study antimicrobial activity of the guanine analogues were checked against five clinical pathogens at the concentrations of (10,20,40,80 µg/ml). The purine compounds showed characteristic Zone of inhibition against tested pathogens. Gentamycin and Fluconazole antibiotics were used as reference compounds.

Table 4: Antimicrobial activity of Guanine analogues

Organisms	***E.coli***	***P. aeruginosa***	***S. aureus***	***Micrococcus***	***Candida albicans***
MIC Values in µg/ml					
Gentamycin	**3.5**	**3.5**	**3.0**	**3.4**	**…….**
Fluconazole					**1.7**
SA-A	**1.7**	**…**	**2.3**	**1.0**	**…**

SA-B	**0**	**1.7**	**1.8**	**…**	**…**
SA-C	**1.4**	**1.8**	**2.1**	**1.6**	
SA-D	**1.4**	**1.9**	**2.1**		**2.6**
SA-E	**2.3**	**1.9**	**2.0**	**1.5**	**3.1**
SA-F	**1.2**	**1.0**	**1.1**	**1.4**	**2.8**
SA-G	**2.3**	**1.5**	**…**	**…**	**…**
SA-H	**1.3**	**2.3**	**0.9**	**…**	**…**
SA-I	**0.9**	**1.5**	**2.3**	**0.8**	**2.6**
SA-J	**1.3**	**2.3**	**…**	**…**	**…**

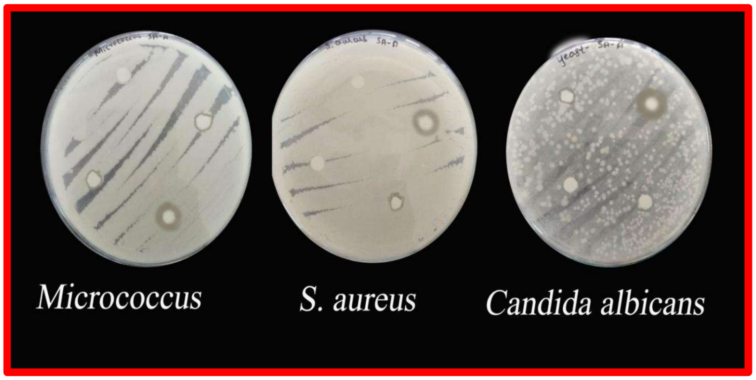


Figure 3a: Antimicrobial activity of guanine analogue (SA-A) on bacterial strains Micrococcus, *S.aureus* and *C.albicans*.

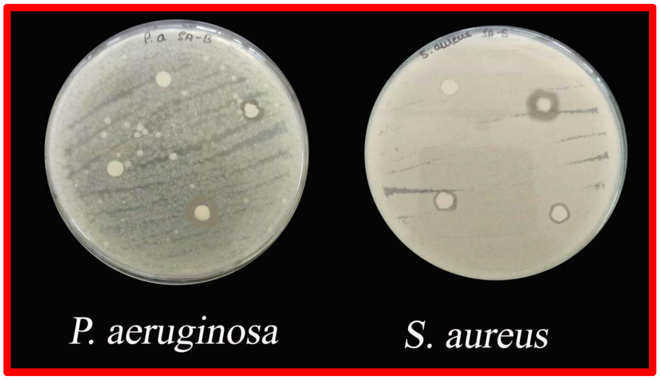


Figure 3b: Antimicrobial activity of guanine analogue (SA-B) on bacterial strains - *P. aeruginosa* and *S.aureus*.

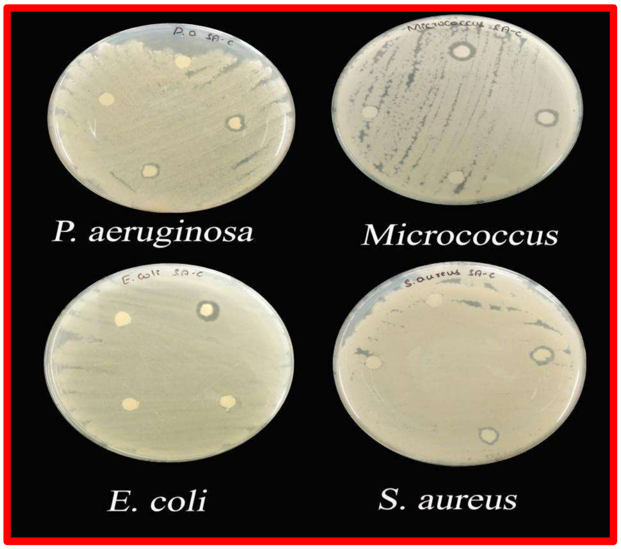


Figure 3c: Antimicrobial activity of guanine analogue (SA-C) on bacterial strain- *E.coli* and *S.aureus*.

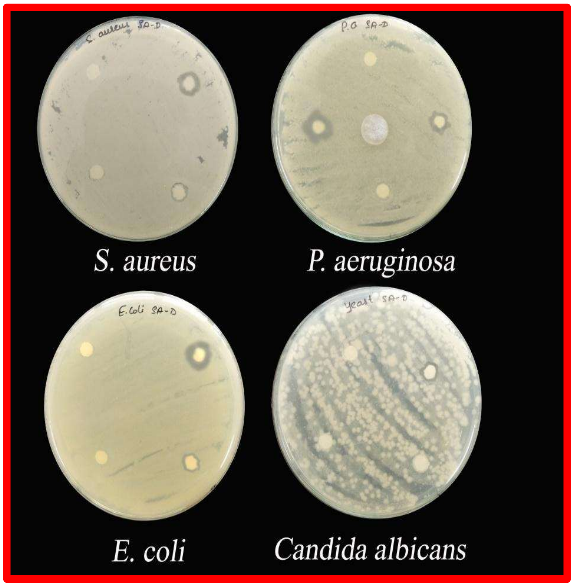


Figure 3d: Antimicrobial activity of guanine analogue (SA-D) on bacterial strains- *S.aureus*. *P. aeruginosa, E.coli* and Micrococcus

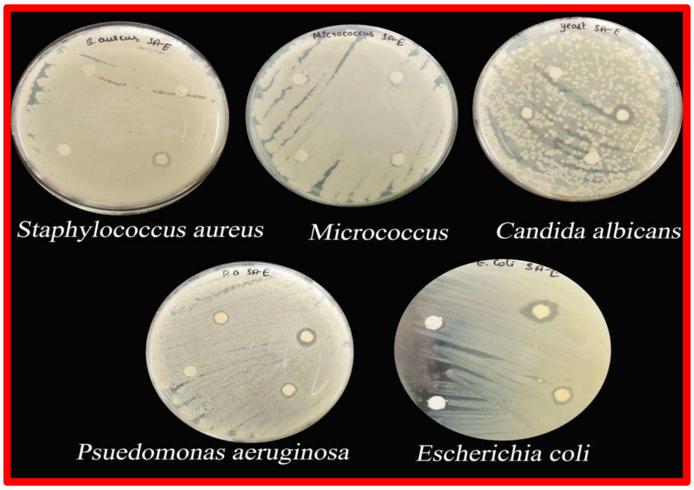


Figure 3e: Antimicrobial activity of guanine analogue (SA-E) on bacterial strains- *S.aureus*. Micrococcus, *C.albicans* , *P. aeruginosa* and *E.coli* .

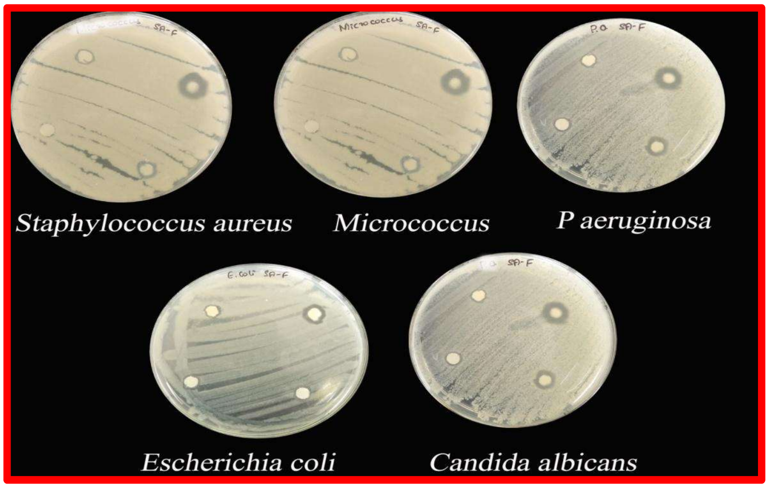


Figure 3f: Antimicrobial activity of guanine analogue (SA-F) on bacterial strains- *S.aureus*. Micrococcus, *P. aeruginosa, E.coli* and *C.albicans* .

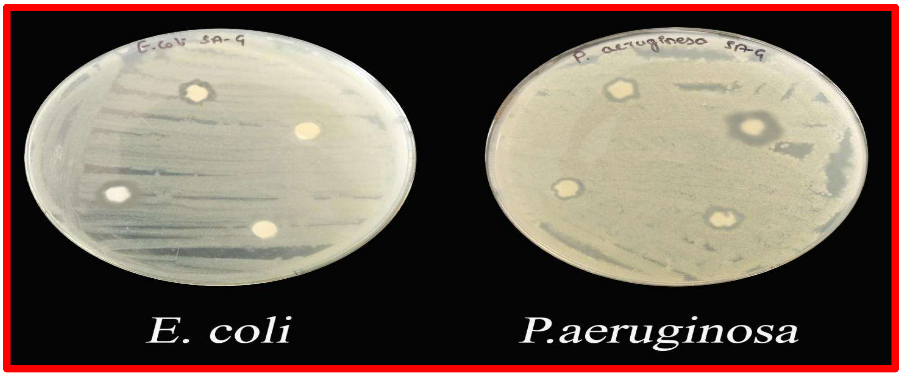


Figure 3g: Antimicrobial activity of guanine analogue (SA-G) on bacterial strains- *E.coli* and *P. aeruginosa.*

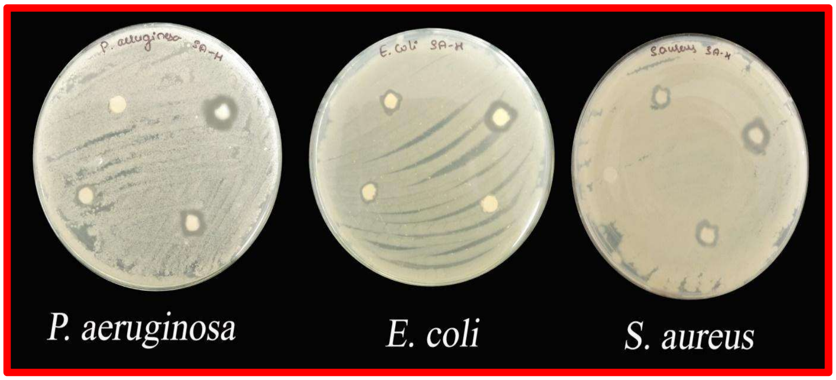


Figure 3h: Antimicrobial activity of guanine analogue (SA-H) on bacterial strains- *P. aeruginosa, E.coli* and *S.aureus.*

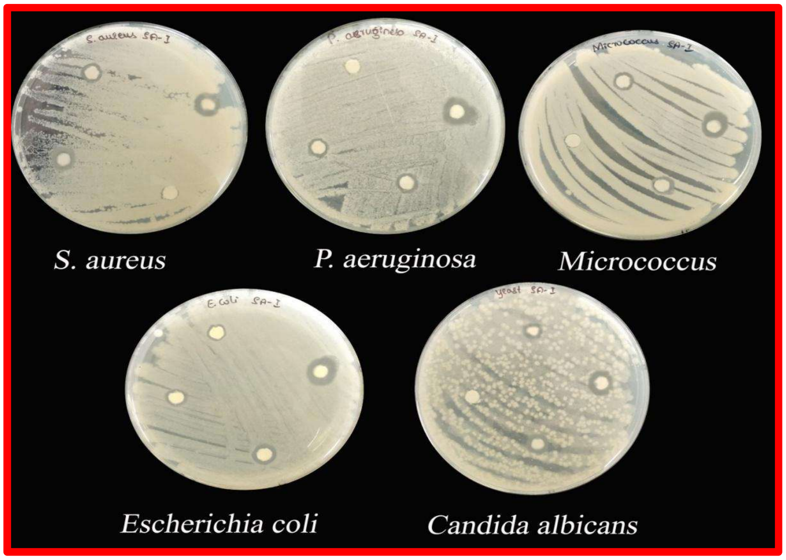


Figure 3i: Antimicrobial activity of guanine analogue (SA-I) on bacterial strains- *S.aureus*. *P. aeruginosa,* Micrococcus, *E.coli* and *C.albicans*.

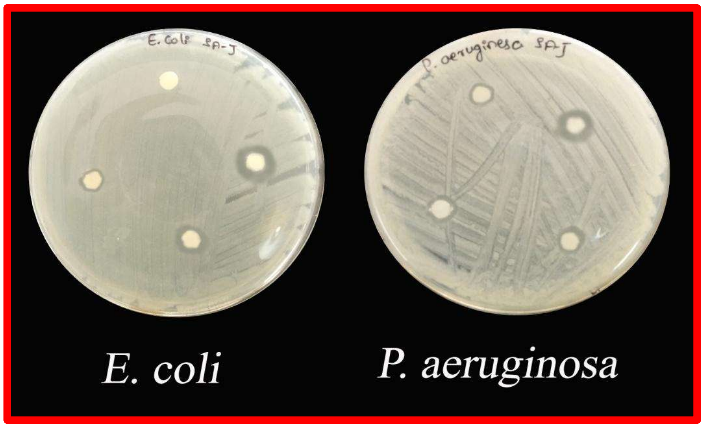


Figure 3j: Antimicrobial activity of guanine analogue (SA-J) on bacterial strains- *E.coli* and *P. aeruginosa*.

Resistance to the number of antimicrobial agents among a variety of clinically significant bacteria is becoming increasingly important. There are various problems arising with the use of antimicrobials such as local tissue irritation, interference with wound healing process, hypersensitivity reaction, system toxicity, narrow antimicrobial spectrum, and emergency of resistance. So, the increasing clinical importance of drug resistant microbial pathogens has additional urgency in microbiological and antifungal research. A wide variety of hetero cyclic systems have been explored for developing pharmaceutically important molecules. Among them the Purine derivatives have been playing an important role in medicinal chemistry.

Compounds (SA-A to SA-J) were tested in vitro for their antimicrobial activity in two Gram-negative and two Gram-positive bacterial strains. Commercial antibiotics such as Gentamycin and Fluconazole were used as standard drugs. The results were compared with standard drugs and depicted in Table 3. Compound SA-E, SA-F, SA-I, were found to be more potent against all the clinical pathogens. The other compounds SA-C, SA-D, exhibited moderate antimicrobial activity against 4 clinical pathogens. SA-A, SA-H, analogues exhibited anti-microbial activity against 3 clinical pathogens. SA-B, SA-G, SA-J were found to be less potent and showed anti-microbial activity against only 2 clinical pathogens.

4. Gastric H^+ /K^+ -ATPase activity.

Drugs of SA-A, SA-D, SA-E, SA-G, SA-J inhibit the activity of ATPase with the inhibition values of 36.6, 40.44, 40.96, 38.85, 35.56 µg/ml respectively; compared to standard (Omeprazole) which had an EC50 value of 27.73 µg/ml. SA-E showed higher potency and SA-J showed least potency.

Table 5: H+/K+ ATPase inhibition activity of guanine analogues

SI NO	Guanine analogues	EC 50 value µg/ml
1	Omeprazole	27.73
2	SA-A	36.6
3	SA-B	No inhibition
4	SA-C	No inhibition
5	SA-D	40.44
6	SA-E	40.96
7	SA-F	No inhibition
8	SA-G	38.85
9	SA-H	No inhibition
10	SA-I	No inhibition
11	SA-J	35.56

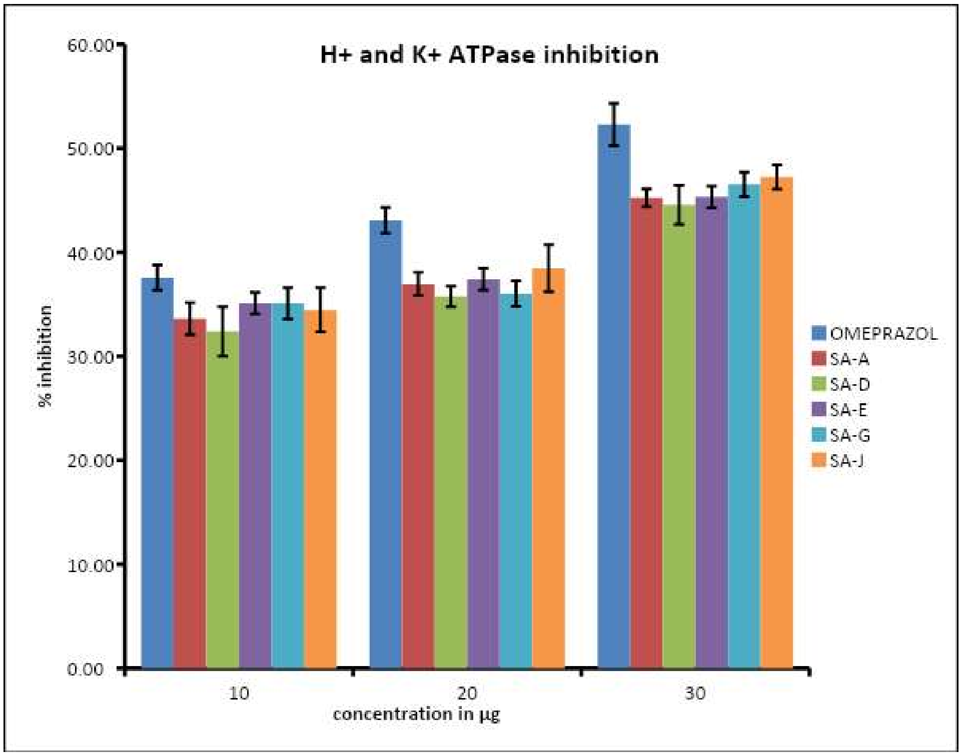


Figure 4: H+/K+ ATPase inhibition activity of Guanine analogues.

5. Alpha-amylase inhibition assay

The Pancreatic α-amylase inhibition of synthesized compounds **SA (A-J)** was studied in terms of oxidation of ketone functional group using DNS method. All the compounds inhibited α- amylase enzyme significantly with an IC_{50} values ranging between 32.25µg/mL and 71.27µg/mL compared to the reference compound acarbose with an IC_{50} value of 95.8 µg/mL **table(3)**.

Table 6: Pancreatic α-amylase inhibition of guanine analogues SA(A-J)

Compounds	**IC_{50} in µg/mL**
SA-A	32.25
SA-B	71.27
SA-C	33.70
SA-D	44.37
SA-E	60.69
SA-F	45.38
SA-G	53.70
SA-H	70.0
SA-I	58.8
SA-J	45.9
Acarbose	95.80

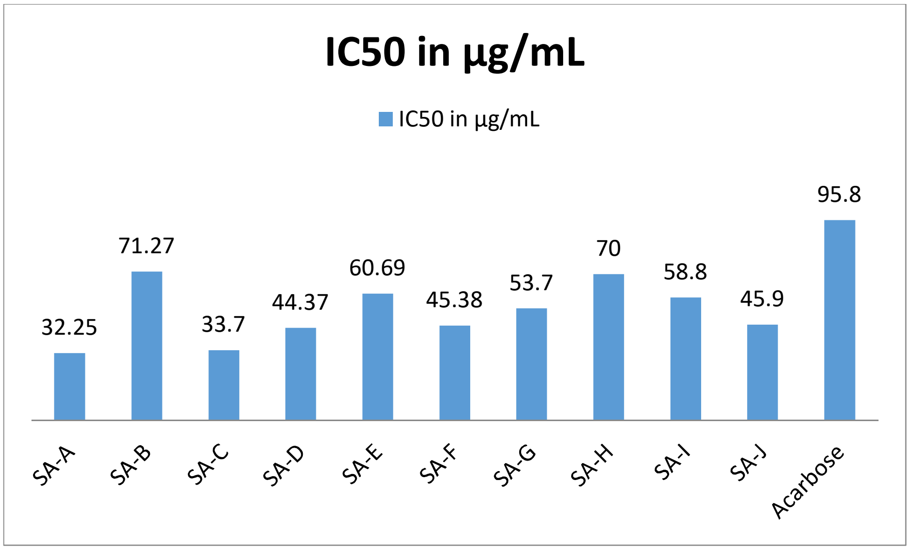


Figure 5: Pancreatic α-amylase inhibition of guanine analogues SA(A-J)

CONCLUSION

The synthesised guanine analogues gave good yield with purity. The biological activity elucidated analogs revealed vital biological activities and the literature analysis has unravelled the great interest from researchers. Results of the present findings are encouraging and all the synthesized compounds when tested showed good antimicrobial, anti-oxidant along with DNA binding and protecting properties. These guanine derivatives will be of great importance since these compounds may prove as potential therapeutic agents for treatment of various infections caused by different microorganisms.

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CONFLICT OF INTEREST

The authors have no competing interest

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