DNA Cleaving Properties of D & L Amino Acid Transition Metal Complexes

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Abstract

This study investigates the synthesis of pure neutral D & L valine and asparagine copper(II) complexes, as well as its DNA cleaving properties on Carolina pGreen DNA and antimicrobial capabilities against *E. coli and B. cereus*. Results obtained suggest greater nuclease activity by the D-valine copper(II) complex compared to its L isomer counterpart. The well diffusion test was conducted to determine the antibacterial capabilities of the D and L-valine copper(II) complexes, while the colony count method was used to determine that of the L-asparagine copper(II) complex. At their respective minimum cleavage concentrations, the valine complexes exhibited no significant antibacterial properties, while a significantly lower number of colonies were formed in the presence of the L-asparagine copper(II) complex at high concentrations.

1. Introduction

Modern chemotherapy is promoted on the basis of metal ions and metal complexes, which have been known to play key roles in the pharmaceutical properties of drugs (Tripathi & Kamal, 2015). The body contains many proteins driven by copper, some of which are important enzymes in metabolism, and in deficiency may cause long term illnesses (Aoki, 2004). Studies such as that conducted by Raman, Raja, & Sakthivel in 2007 have suggested that amino acid-Schiff base metal complexes do possess significant DNA cleaving and antibacterial properties, and previous literature show that amino acid metal complexes can be synthesized using DL, L and D variants of each amino acid (Abdul et al., 2014). However there is a dearth in the investigation of the DNA cleaving capabilities of amino acid metal complexes, as well as the possible differing nature of the extent of nuclease activity and antibacterial capabilities of amino acid and amino acid-Schiff base metal complexes synthesized by pure amino acids of different isomer types may possess. Thus this study aims to investigate the nuclease capabilities of amino acid metal complexes synthesized by pure amino acid isomer types, to determine if there is a natural difference in the nuclease activity and antibacterial properties of the amino acid metal complexes.

Methods of synthesis range from using acidic to alkali medium (Abdul et al., 2014), however it is more favoured amongst literature to synthesize the complex in an alkali medium as seen in studies such as that of Stanila, Braicu, Stanila, & M.Pop in 2011. Most studies obtain solid complexes that precipitate out of the reaction mixture naturally, but it can also be inferred

from the antibacterial tests reported that the complexes are soluble in either water or DMSO. The complexes formed can either be an uncharged molecule, or a charged cation to the anion present in the solution. Previous literature seem to disagree on the type of complexes that is formed (Tripathi & Kamal, 2015)(Abdul et al., 2014), showing that there is still a void in the understanding of the mechanisms behind the synthesis of amino acid metal complexes. On both sides of the issue, elemental analysis was used as the main form of characterization. While other forms of analysis such as FTIR (Marcu et al., 2007), UV-vis spectroscopy and NMR (Sharma & Dubey, 1993) have also been conducted, it seems to not be as conclusive in the characterization of synthesized complexes.

The presence of free radicals has also been suggested to play a major role in facilitating the nuclease activity of the complexes. Small amounts of amino acid-Schiff base metal complex-hydrogen peroxide mixtures at low concentrations have been shown to possess significant nuclease capabilities (Raman et al., 2007), and it is possible that amino acid metal complexes may possess similar properties due to its similar general structure, as well as the similar purposes the ligands serve in controlling the cleaving properties of the ionic metal center.

2. Objectives and Hypothesis

This study aims to investigate the synthesis and DNA cleaving properties of pure D & L-valine and asparagine copper(II) complexes, as well as to determine the antimicrobial capabilities of the complex formed.

It is hypothesized that the D & L-valine and asparagine copper(II) complexes can be synthesized using the methods outlined below, the complexes formed will show DNA cleaving and antibacterial properties, and that the complexes formed with differing amino acid isomer types will show differing DNA cleaving and antibacterial properties, with one isomer type invariably outperforming the other.

3. Outline of Methods

Synthesis of valine copper(II) complex

2 mmol of D-valine was dissolved in 20ml of deionized water, and 1.15 mmol of crushed $Cu(OH)_2$ was added to the valine solution. The mixture was stirred for 4 hours and filtered. The filtrate was recrystallized to form the complex. The steps were repeated with L-valine to obtain the L-valine copper(II) complex. CHNS and electro-conductivity analysis was conducted to characterize the complexes formed.

Synthesis of asparagine copper(II) complex

2 mmol of sodium acetate was dissolved in 20ml of deionized water. 2 mmol of D-asparagine was added to the solution and stirred. 1.15 mmol of $CuSO_4$ dissolved in 5ml of water was

added dropwise to the reaction mixture, and left to stir for 4 hours. The mixture was filtered and the residue washed and left to dry. The above steps were repeated with L-asparagine to obtain the L-asparagine copper(II) complex. CHNS analysis was conducted to characterize the complexes formed.

DNA Cleavage Test without the use of hydrogen peroxide

5µl of dissolved D & L-valine copper(II) complexes of varying concentrations were added to 10µl of Carolina pGreen DNA and 5µl of deionized water to form each test setup. 10µl of deionized water was added to 10ul of Carolina pGreen DNA to form the control setup, and 5µl of diluted $CuSO_4$ solution was added to 10µl of Carolina pGreen DNA and 5µl of deionized water to form the Cu^{2+} control setup. 5µl of 1kb Promega DNA ladder was used as the DNA ladder. 2.5µl of DNA electrophoresis sample loading dye and 1.5µl of SYBR Green I was added to the ladder, tests and control, and the mixtures centrifuged for 5 seconds at 2000rpm before being loaded onto 1% agarose gel. The samples were electrophoresed on the gel using *tris*-acetic acid-ETDA buffer solution at 100V for 45 minutes until the dye was 75% down the gel. Afterwards the gel was shone under ultraviolet light to allow the DNA bands to be visible. The presence of DNA cleavage was determined by significant difference between the bands formed by the test setup and the control setup.

DNA Cleavage Test with the use of hydrogen peroxide

5µl of dissolved D & L-Valine copper(II) complexes of varying concentrations were added to 10µl of Carolina pGreen DNA, together with 1µl of 10mM hydrogen peroxide solution and 4µl of deionized water to form each test setup. 10µl of deionized water was added to 10µl of Carolina pGreen DNA to form the control setup without hydrogen peroxide. 9µl of deionized water was added to 1µl of 10mM hydrogen peroxide solution and 10µl of Carolina pGreen DNA to form the control setup with hydrogen peroxide. 5µl of 1kb Promega DNA ladder was used as the DNA ladder. Loading dye and SYBR Green I was added, and the mixtures centrifuged and loaded onto the agarose gel. The samples were electrophoresed on the gel using *tris*-acetic acid-ETDA buffer solution at 100V for 45 minutes until the dye is 75% down the gel.

To confirm the DNA cleaving properties of the dissolved D & L-Valine copper(II) complexes in the presence of free radicals, 5µl of dissolved D & L-Valine copper(II) complexes at the minimum cleaving concentration was added to 10µl of Carolina pGreen DNA, together with 1µl of 10mM hydrogen peroxide solution, 1µl of 20mM ascorbic acid solution and 3µl of deionized water to form the test setup with ascorbic acid. The test setups with hydrogen peroxide, control setup and control setup with hydrogen peroxide were prepared the same

way as before. Loading dye and SYBR Green I was added, and the mixtures centrifuged and loaded onto agarose gel. The samples were electrophoresed the same way as before, with the gel being shone under ultraviolet light afterwards.

The presence of DNA cleavage by the complexes in the presence of free radicals was determined by significant difference in the bands formed by the test setups with hydrogen peroxide to the band formed by the control setup with hydrogen peroxide and the test setup with ascorbic acid.

Growth of Bacteria

Bacillus cereus ATCC 11778 *& Escherichia coli* ATCC 25922 were grown in 10ml of LB broth overnight at 30℃ with shaking. The absorption of bacterial cultures at 600 nm was measured to determine cell density and standardize bacterial population.

Well Diffusion Test

Each bacterial culture was swabbed evenly on a Mueller-Hinton agar plate. 4 wells were created in the agar. 100µl of the dissolved valine copper(II) complex solution at its minimum cleaving concentration with 0.5mM of hydrogen peroxide was added to a well. The positive control was 10% bleach and the negative control was sterile water. The hydrogen peroxide control was 0.5mM hydrogen peroxide solution. The plates were incubated at 30℃ overnight and the diameter of the zone of inhibition determined the next day.

Colony Count Test

The test setup was prepared by adding 0.5ml of bacterial culture to 5ml of a suspension of 0.1M L-asparagine copper(II) complex and 4.5ml of 2x LB broth. The control setup was prepared by adding 0.5ml of bacterial culture to 9.5ml of LB broth. The mixtures were incubated at room temperature for 2 hours. Serial 10-fold dilutions were done with 0.85% sodium chloride solution and 0.1ml of the diluted culture was spread on the LB agar using a sterile spreader. The plates were incubated at 30℃ overnight. The number of colonies was determined the following day.

4. Results and Discussion

Synthesis of valine copper(II) complexes

The reaction mixture immediately turned from colourless to deep blue upon the addition of crushed copper(II) hydroxide. The filtrate formed royal blue crystals upon crystallization. CHNS results as shown in Fig. 1 suggest that the complexes formed can take two possible forms, either as charged, unhydrated cations, or uncharged, hydrated molecules. An electro-conductivity test was conducted by dissolving a few crystals in deionized water, and measuring its electro-conductivity in microsiemens. Given the absence of significant amounts

of valine impurities as shown in Fig. 1, the results in Fig. 2 suggest that the proportion of uncharged complex molecules far outweigh that of the charged complex cations due to the significant increase in electro-conductivity that a relative increase in the yield of charged complex cations would result in. Thus it was inferred that the main product of synthesis was the uncharged, hydrated form of the complex.

Complex	Molecular weight	C%	H%	N%
% Found	**-**	**36.56**	**6.66**	**9.08**
$Cu(Val)_2.2H2O$	**331.5**	**36.20**	**7.24**	**8.45**
***$[Cu(Val)_2](OH)_2$**	**331.5**	**36.20**	**7.24**	**8.45**

Fig. 1, *it is understood that in this form of the complex, valine exists as $C_5H_{12}NO_2$ instead of its typical form of $C_5H_{11}NO_2$.

	Complex sample	Copper(II) control	Valine control	Deionized water
Conductivity uS/cm	**79.9**	**440**	**3.77**	**1.30**

Fig. 2, the electro-conductivity of the complex sample is significantly less than that of the $CuSO_4$ control at an equal concentration of Cu atoms.

Synthesis of asparagine copper(II) complexes

The reaction mixture immediately turned from colourless to royal blue upon the addition of aqueous copper(II) sulfate. After a period of time, light blue precipitate started to form, which increased in concentration as the reaction progressed. Residue obtained after filtering turned faint bluish-purple as it dried. CHNS analysis in Fig. 3 suggests that the residue formed was the neutral anhydrous asparagine copper(II) complex. It can thus also be concluded that the asparagine copper(II) complex is insoluble in water.

Complex	Molecular weight	C%	H%	N%	S%
% Found	**-**	**28.80**	**4.33**	**16.68**	**<0.50**
$Cu(Asn)_2$	**325.74**	**29.47**	**4.30**	**17.19**	**-**

Fig. 3, the % compositions of the theoretical complex and the actual composition of the complex formed are very similar.

Theory behind the synthesis of complexes

Soluble amino acids such as valine may exist in three dominant forms. Based on the equation $[HA] = [A^-]$ when pH = pKa, given that the pKa of the carboxylic acid group is 2.32 and that of the amine group is 9.62, when the pH of the reaction mixture is less than 2.32, the form as

shown by Fig. 4 will be proportionally dominant. When the pH of the reaction mixture is greater than 2.32 but less than 9.62, the form as shown by Fig. 5 will be proportionally dominant, and likewise when the pH is greater than 9.62, the form as shown by Fig. 6 will be proportionally dominant. In order to form bonds with the metal cation, the functional groups of the amino acid must have available lone pairs to conduct electrophilic attack. Thus an amino acid with saturated functional groups such as that shown in Fig. 4 will be unable to form bonds with metal cations, while that of Fig. 5 and Fig. 6 will form bonds as shown in Fig. 8 and Fig. 7.

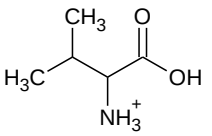

Fig. 4, pH < 2.32

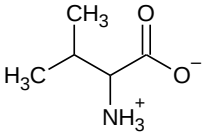

Fig. 5, 2.32 < pH < 9.62

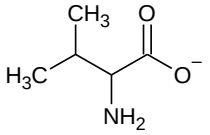

Fig. 6, pH > 9.62

As the type of complex formed is heavily influenced by the form of the amino acid, it can be inferred, for soluble amino acid metal complexes, that the pH of the reaction mixture is the determinant of the type of complex formed. Ideally the pH of the reaction mixture should be greater than 9.62, however reaction mixtures with such a high concentration of OH^- will form large amounts of $Cu(OH)_2$, greatly reducing the yield of the complex formed. While it is possible to obtain potentially a much purer filtrate of the uncharged valine copper(II) complex at pH 9.62, it was not investigated in this study due to the difficulty in removing impurities that a basic reagent would add to the reaction mixture, as well as concerns of very low yield. As the valine copper(II) complexes formed are soluble, with its form being dependent on the pH of the solution, it is possible that the synthesis of the soluble amino acid complexes is also influenced by the equilibrium between the valine and Cu^{2+} reactants, the singly chelated complex intermediate, as well as the doubly chelated products of the reaction. This is illustrated in Fig. 7 and Fig. 8.

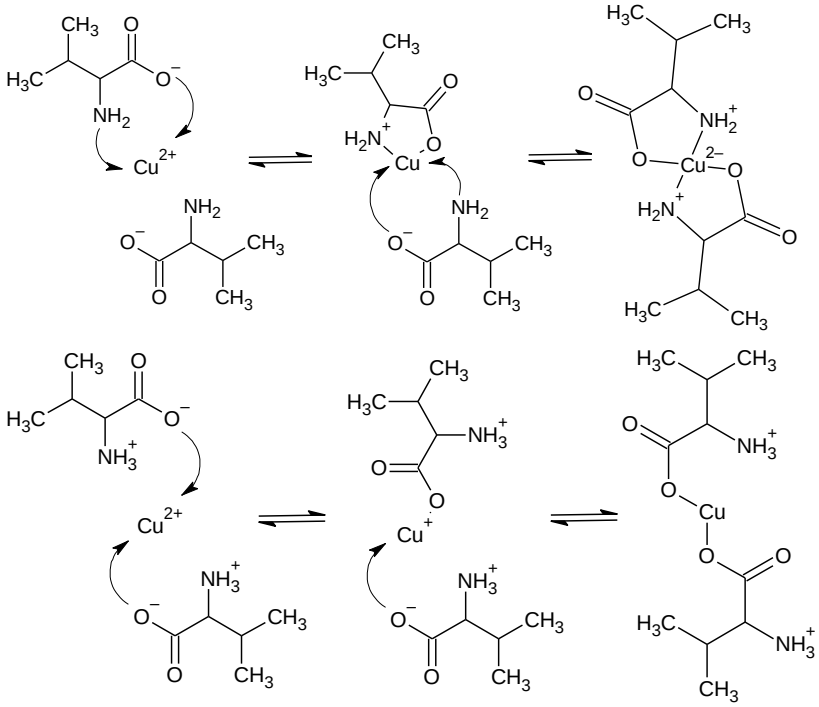

Fig. 7, as both the amine and carboxylic acid groups are deprotonated, lone pairs on both groups are available to conduct nucleophilic attack, thus both functional groups coordinate with the Cu^{2+} center. The formation of the $[Cu(Val)]^+$ intermediate complex is required before the formation of the uncharged $Cu(Val)_2$ complex can occur, it shows how the uncharged $Cu(Val)_2$ complex can by itself form charged molecules when dissolved in water.

Fig. 8, only the carboxylate acid groups are able to coordinate with the Cu^{2+} center as the amine groups are saturated. The formation of the $[Cu(Val)]^{2+}$ intermediate complex is required before the formation of the charged $[Cu(Val)_2]^{2+}$ complex can occur.

For the synthesis of insoluble amino acid metal complexes such as asparagine copper(II) complexes, there is not a need to control the pH as soluble chemical species can be added to the reaction mixture to artificially deprotonate the amino acid used without contaminating the product formed at the end of the reaction due to the insoluble nature of the complexes. Thus reaction mixtures for such insoluble complexes after the addition of the metal salt can have very low pH values, but still a high yield.

DNA Cleaving test without the use of hydrogen peroxide

There is a slight difference in the bands formed by the sample and pGreen control at high sample concentrations, suggesting that the L-valine copper(II) complex alone may possess minor nuclease activity at high concentrations. This is illustrated in Fig. 9.

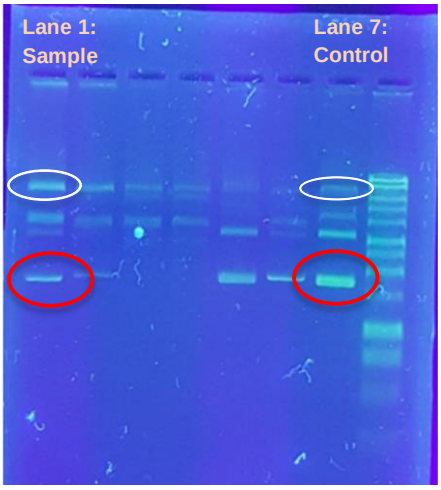

Fig. 9, lane 1 contains the complex with pGreen DNA while lane 7 contains pGreen only.

The bands circled in red most likely represent the movement of the supercoiled pGreen DNA. It can be seen from the intensity of the bands that there is less supercoiled and more open-circular DNA (indicated in white) present in the sample compared to the control setup. It can also be seen that more linear DNA strands of around 4500 bases, and less of that around 3000 or 5000 bases are present, thus suggesting that nuclease activity was present, with the complex possibly possessing the ability to cleave DNA at multiple restriction sites. The exact concentration of the complex solution used was unable to be determined as the immediate filtrate of the reaction mixture was used to maximize the concentration of the complex solution due to limitations in the dissolution of complex crystals. Further study on the nuclease activity of the complex alone was not conducted due to the infeasibility in using high concentrations of the metal complex in applications as a result of its toxicity to bacterial and human cells.

DNA cleaving test with the use of hydrogen peroxide

With the addition of hydrogen peroxide, the difference in the bands formed by the L-valine samples and the control with hydrogen peroxide is insignificant below a complex concentration of 0.5mM. However at concentrations of 0.5mM or greater, the complex exhibits significant nuclease activity, most likely eliminating the band of supercoiled pGreen DNA by cleaving supercoiled DNA into open-circular or linear strands, and producing its own distinct band (circled in white). This is illustrated in Fig. 10.

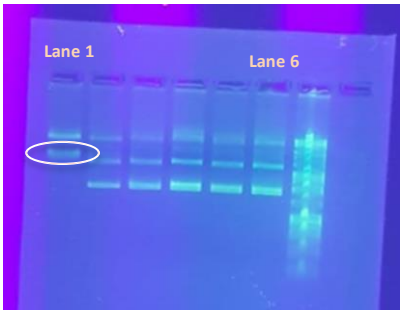

Fig. 10, lanes 1-5 are the samples, lane 6 is the control.

Lanes 1-5 contain complex concentrations of 0.5mM, 0.4mM, 0.3mM 0.2mM and 0.1mM respectively. Bands formed in lanes 2-5 are similar to the control (Lane 6), while the bands formed in lane 1 are dissimilar to all the rest. The lack of bands after the distinct band (in white) can be interpreted as what may be the occurrence of random cleavage, it can thus be inferred that the complex at a concentration of 0.5mM is likely able to cleave DNA at multiple restriction sites. It was concluded that the minimum significant cleaving concentration of the L-valine complex in the presence of free radicals is 0.5mM. In the case of D-valine, the bands formed by samples and the control seem to differ at much lower complex concentrations, at which some of the bands formed in the control are not present in the samples. It is the bands formed in lanes 1 & 2, containing complex concentrations of 0.1mM and 0.09mM respectively, that stand out from the rest of the gel. This is illustrated in Fig. 11. The near complete disappearance of the bands representing linear strands of around 4000 and 5000 bases (circled in white in lanes 5 and 9 for illustrative purposes), together with the dimmer band representing supercoiled pGreen DNA, possibly suggests that the D-valine copper(II) complex is able to cleave pGreen DNA at multiple restriction sites. It was concluded that the minimum significant cleaving concentration of the D-valine complex in the presence of free radicals is about 0.09mM. Based on the data obtained alone, it is unable to be determined if the cleavage is random.

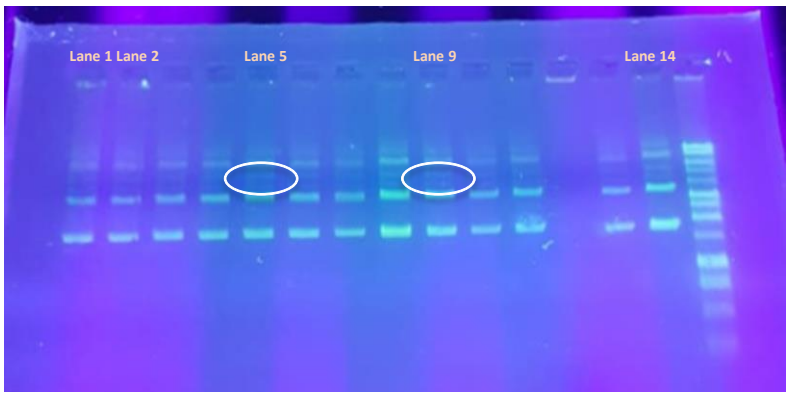

Fig. 11, lanes 1-10 contain samples with complex concentrations ranging from 0.1mM to 0.01mM with a division of 0.01mM between each well. Lane 14 shows the bands formed by the pGreen control setup with hydrogen peroxide.
*Image may not accurately depict the bands formed due to poor image quality.

To confirm the nuclease activity observed in Fig. 11, an excess of ascorbic acid was added to the samples to remove the hydroxyl radicals. The resulting bands that formed were near identical to the pGreen control without hydrogen peroxide (Fig. 12), thus it can be inferred that the main mechanism behind the nuclease activity of D and L-valine copper(II) complexes is likely one with significant use of free radicals. It can also be concluded that the D and L-valine copper(II) complexes may differ in their DNA cleaving mechanism given the difference in the type of bands formed, and that the D-valine copper(II) complex may have more significant DNA cleaving properties than its L isomer counterpart.

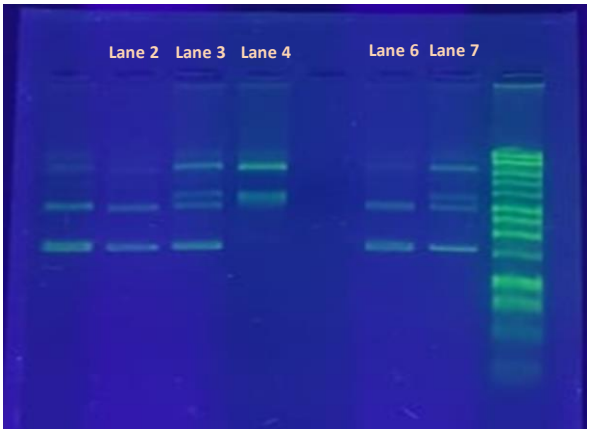

Fig. 12, lane 2 contains the test setup for the D-valine copper(II) complex with hydrogen peroxide, lane 4 contains the test setup for the L-valine copper(II) complex with hydrogen peroxide, lane 3 contains the test setup for the complexes with hydrogen peroxide and ascorbic acid, lane 6 contains the pGreen control with hydrogen peroxide, and lane 7 contains the pGreen control without hydrogen peroxide.

Well diffusion test

The zones of inhibition around the wells containing the complexes with hydrogen peroxide were minimal or absent, thus it was inferred that the valine copper(II) complexes do not possess significant antibacterial properties at their respective minimum cleavage concentrations. It is hypothesized that a cause behind the absence of zone of inhibitions may be the extremely low concentration of the complex and hydrogen peroxide, resulting in the bacterial cells being able to resist damage to its genome by preventing significant amounts of the complex from permeating through its cell membrane, and or by metabolizing the small amounts of hydroxyl radicals present.

Colony count test

As shown in Fig. 15, there is a clear decrease in the number of colonies formed for *E. coli* in the presence of the L-asparagine copper(II) complex. The P-value for this Mann-Whitney U test was 0.030, which is less than 0.05, hence the data suggests that the complex synthesized was effective in the inhibition of the growth of *E. coli*.

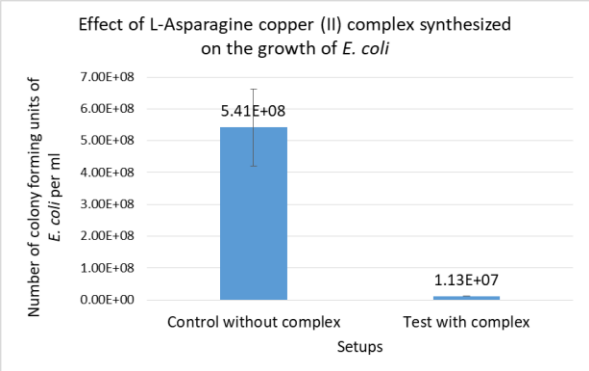

Fig. 13, the graph shows the effect of the L-asparagine copper(II) complex on the number of colonies formed.

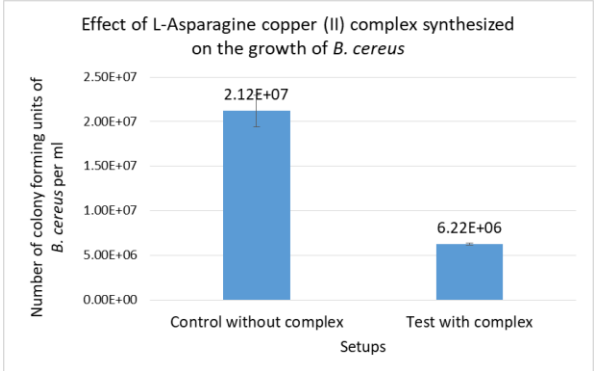

Fig. 14, the graph shows the effect of the L-asparagine copper(II) complex on the number of colonies formed.

Fig. 16 depicts the decrease in the number of colonies formed for *B. cereus* in the presence of the L-asparagine copper(II) complex. The P-value for this Mann-Whitney U test was 0.012, which is less than 0.05, hence the data suggests that the complex synthesized was effective in

inhibiting the growth of *B. cereus*. The reduced relative difference in colonies formed between test and control setups may be due to the differences in cell wall structure between gram-negative and gram-positive bacteria, resulting in gram-positive bacteria being more resistant to physical disruption.

5. Conclusions and recommendations for future work

In conclusion, pure L and D-valine and asparagine copper(II) complexes were synthesized and characterized using CHNS analysis. To more conclusively determine the type of complex formed, X-ray crystallography could be conducted to determine the structure of the complex molecules. The soluble valine complexes exhibited substantial DNA cleaving properties at low concentrations in the presence of free radicals, while also exhibiting nuclease activity by itself at high concentrations. The minimum significant cleaving concentration of the D-valine complex is less than that of the L-valine complex, thus suggesting the possibility that the D-valine complex has greater DNA cleaving capabilities than its L isomer counterpart. However, the nuclease activity of the D-valine complex at greater concentration was not investigated. It is possible that the D-valine complex cleaves DNA using the same mechanism as its L isomer counterpart, and that the minimum cleaving concentration that was found is inaccurate. Further experimentation is required to determine if the hypothesis is true. Studies can also be conducted to conclusively deduce the nuclease activities of the complexes using more accurate testing methods such as the use of purer DNA, as the methods used in this investigation cannot confidently determine the source and effect of the observed nuclease activity, thus resulting in only interpretations of the observations but no conclusive findings. No significant antibacterial activity was observed for either complexes at their respective minimum cleaving concentrations in the presence of free radicals. Further study could be conducted by increasing and standardizing the concentration of complex solution used to conclusively compare the antibacterial properties of the D and L isomers of the complex. Different amino acids could also be used to develop a possible trend on the difference in DNA cleaving and antibacterial properties of the complexes of D and L isomers. Likewise, studies on the DNA cleaving and antibacterial properties of amino acid-Schiff base metal complexes of D and L isomers can also be conducted to develop deeper understanding into the mechanisms behind its nuclease activity and antibacterial capabilities. The insoluble L-asparagine complex exhibited antibacterial activity against *E. coli* and *B. cereus*, but more significantly against *E. coli*. Methods should be developed to allow such complexes to dissolve in solvents to increase its range of applications to include areas such as chemotherapy.

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