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In vitro Antifungal and *In silico* Antibacterial Evaluations of Anacardic Acid and its Complexes from Cashew Nut Shell Oil

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ABSTRACT

Anacardic Acids (AcA) have been documented to have particularly important pathogenic properties. AcA and its complexes are being investigated in this article for their antimicrobial activities through *in vitro* and *in silico* assay methods. AcA from the cashew nut shell oil (CNSO) was isolated and Fourier-transform infrared spectroscopy (FTIR) and Proton nuclear magnetic resonance (^{1}HNMR) were used to characterise the compound. AcA complexes were synthesised with hydrated metal (II) ions of Cobalt and Copper in a ratio of 1:1 using a modified method by Mendes *et al*. The antifungal activities of these AcA metal complexes were tested against five fungi. While AcA showed no antifungal activity, its complexes showed interesting results. Anacardic acid complex of cobalt (AcA-Co) showed activities against three of the five fungi; the best activity was against *Penicillium citrinum* (30±7.07 mm); a better result than clotrimazole (12.5±0.71 mm) which was the reference standard drug used. Anacardic acid complex of copper (AcA-Cu) also showed activities against all fungi tested against except *Aspergillus flavus* with the best activity also against *Penicillium citrinum* (15±2.83 mm). Standard precision (SP) and extra precision (XP) docking of AcA with 1Y54 revealed that it inhibits *Enterococcus spp.* better than standard drugs (Streptomycin and Tetracycline) as shown by docking scores and degree of binding affinities. These results of AcA and its complexes suggest additional studies which could lead to the development of new antifungal and antibacterial agents.

Keywords: Anacardic acid, Antibacterial, Antifungal, Anacardic acid complexes.

Introduction

Antimicrobial infections are quite common and antimicrobial resistance (AMR) organisms are on the increase. The anacardic acid derivatives with alkyl side chain of C_{10} and C_{12} have been reported to show good antibacterial activity and demonstrated multi resistant bacterial strains *in vitro* and *in vivo*.[1] Lima *et al.* reported strong bactericidal activity of zein nanoparticles containing anacardic acid which also showed a long-lasting inhibitory activity against biofilm formation in a *Streptococcus mutans* biofilm model.[2] Anacardic acid is the most abundant of cashew nut shell oil (CNSO) extraction (when extracted naturally). It accounts for 60-65% of the CNSO.[3] Anacardic acid constitute 70% of the CNSO, 8% cardol, and 5% cardanol, with the remainder being made up of other phenols and less polar substances.[4] Depending on the conditions of the roasting process, the composition of the technical CNSO can change and have higher cardanol content (83-84%), less cardol (8-11%) and maintain polymeric material at 10% and 2-methyl cardol content at 2%.[5-7] Some continents like Africa, Asia and South America have long demonstrated using phenolic isolates from cashew nut shell liquid as traditional medicines.[8-10]

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Some uses include antidiarrheal, anti-asthmatic, astringent, tonic medication and depurative. Mouse models were also used by Gomes Júnior *et al*.[11] to demonstrate the antinociceptive, antioxidant and anti-inflammatory activities of AcA. Marinho *et al*. combined both molecular docking and molecular fractionation with conjugate caps methods to establish a more reliable quantitative information on the interaction of AcA (with saturated side chain) and the active site of Trypanosoma cruzi glyceraldehyde-3-phosphate dehydrogenase.[12] The use of anacardic acid as a potential supplementary vitamin for pancreatic cancer prevention and progression was suggested.[13] The research provided data proving the inhibition of pancreatic cancer cell growth through the Chmp1A increase and the activation of ATM and p53. In another research, several pharmacological activities of Anacardic acid were demonstrated. These include anti-inflammatory, anticholinesterase, and antioxidants.

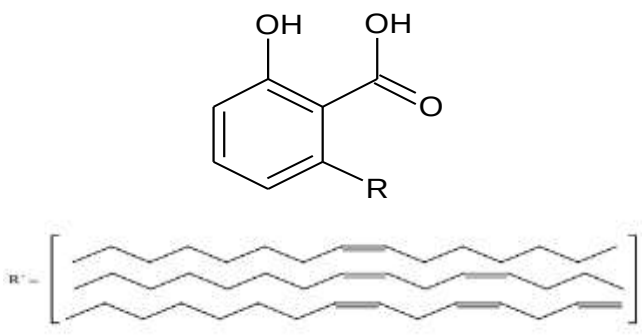

Figure 1: Structure of Anacardic acid

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These are related to the protection against aging disorders. The team also proved that the metals complexes of zinc and copper are co-factors of antioxidant enzymes and can improve brain-protective action by their association with AcA.[14]

In 2018, Schultz *et al*.[15] reported the transcriptomic response of breast cancer cells to anacardic acid. The research focused on identification of the comprehensive consequence of anacardic acid on the RNA transcriptome of two well-characterized breast cancer cell lines representing luminal A, ERα+ (MCF-7) and TNBC (MDA-MB-231).

Antimicrobial resistance (AMR) develops as parasites, viruses, fungi, and bacteria evolve over time and begin to respond less or not at all to treatments and therapies, making it more difficult to cure infections and, as a result, increasing the spread of diseases and, in some cases, causing mortality. Antimicrobial resistance has been adjudged to cause approximately seven hundred deaths annually worldwide and this affects most countries worldwide. This figure has the potential to grow to ten million per year by the year 2050.[16] While Clotrimazole, miconazole, and other antifungal drugs have been successfully used to treat diverse types of fungal infections[17], Imipenem, Sulphamethoxaxole Trimethoprim (SXT), streptomycin and other antibacterial drugs have also been used as standard drugs over time. There is need for development of new and effective antibacterial drugs[18] and to support this assertion, WHO has prioritised the 12 most serious drug-resistant bacteria pathogens for which the development of novel antibacterial treatment options is most needed. Only 8 of the 51 new antibiotics and biologicals identified in the report are considered by the WHO to be novel medicines that will improve the present antibiotic therapy portfolio.[19] Nature has served as the primary source of inspiration for the development of novel antibiotics ever since the early days of antibiotic development. More than 60% of all antibiotics available on the market are natural products or equivalents of natural products.[20] The launch of the AMR Action Fund by the International Federation of Pharmaceutical Manufacturers and Associations, recently, which was boosted by about 1$ billion for research directed towards the objective of providing patients access to two to four new antibiotics by 2030.[21]

This research is therefore aimed at reducing the trend of microbial infections by using readily available (and modified) natural product source (CNSO). The work describes the isolation and characterisation of AcA and its Co^{II} and Cu^{II} metal complexes, their *in vitro* antifungal and *in silico* antibacterial assays with focus on possible new agents for potential actions against their infections.

Materials and Methods

Sample Preparation

Dried cashew nuts were obtained from Ejigbo Local Government area of Osun State, Southwest Nigeria in April 2019 and were identified at Bowen Herbarium with voucher no BUH031. The nuts were further sun-dried for two weeks and were separated from the shells manually using a plier and a knife. The separated shells were further sun-dried for two days before they were pulverized with grinding machine for easier extraction surfaces. The pulverized shells (789 g) were soaked in methanol (1.5 L) for three days to extract the oil (CNSO), which was then concentrated using rotary evaporator and left in the hood until usage.

Isolation of Anacardic acid

Anacardic acid was isolated using the salting-out process.[22] The extracted shell liquid (50 g) was dissolved in 200 mL ethyl acetate and the solution was shaken with 200 mL 10% sodium hydrogen carbonate ($NaHCO_3$). The mixture was allowed to settle in a separating funnel overnight to form two layers (aqueous and organic). The aqueous layer was acidified with 10% hydrochloric acid and transferred into a separating funnel. The anacardic acid formed was extracted with (200 mL x 3) ethyl acetate, dried with magnesium sulphate and concentrated to give a semi solid deep brown phenolic acid with melting point range of 91-94^{o}C (anacardic acid 7 g) which was validated by FTIR and ^{1}HNMR (Figures 2 and 5a).

Complexes Formation

Metal complexes of the isolated AcA were synthesised with copper sulphate pentahydrate and Cobalt chloride hexahydrate using an already established method with little modification.[23]

For AcA-Co; AcA (0.8 g) was dissolves in methanol (10 mL) before Co^{II} (0.3 g) was added and stirred for 90 minutes. During the reaction, the pH of the solution was raised to about 11 with NH_4OH at the temperature of 30 ^{o}C. The solution was allowed to cool down to room temperature, filtered and washed with 10 mL methanol. The residue (complex) was then dried in desiccator and weighed to give 0.14 g black powder (12% yields). Similar procedure was used for AcA-Cu synthesis except that 0.57 g of Cu^{II} was used and the temperature for the reaction was raised to 55 ^{o}C to give a brown powder 0.58 g (42% yields). Agilent Technologies Cary 630 FTIR, Agilent Technologies 400MHz NMR and Schimadzu UV-1800 were used for the characterisation of the AcA and its complexes.

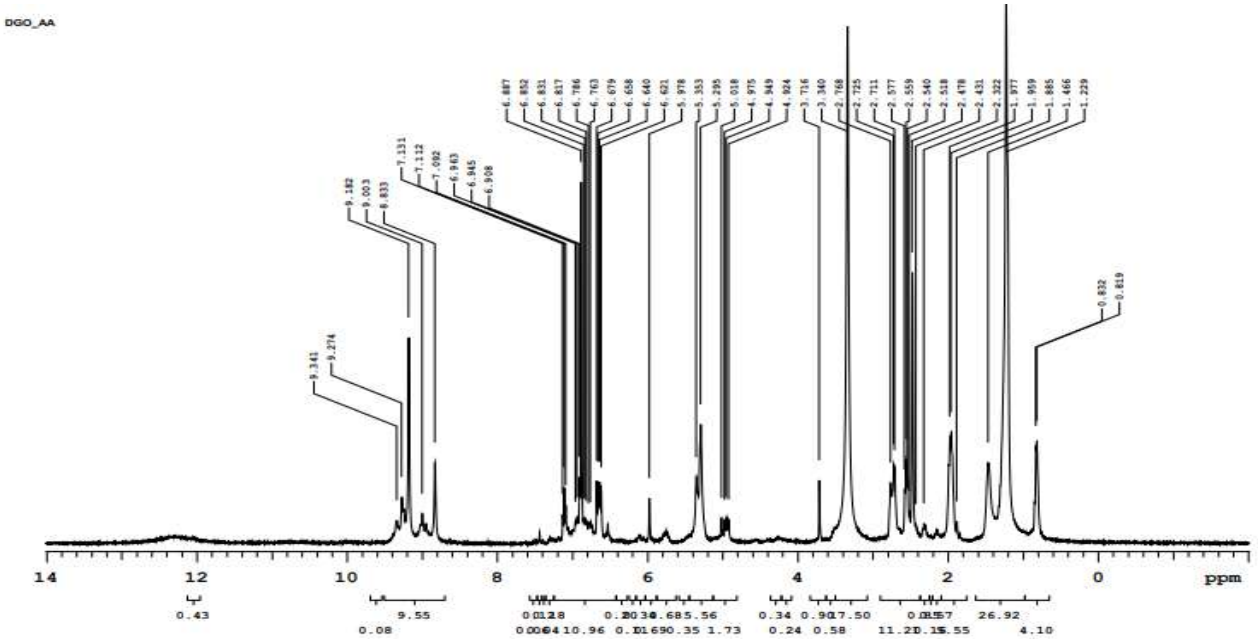

Figure 2: ^{1}H-NMR Spectrum of anacardic acid

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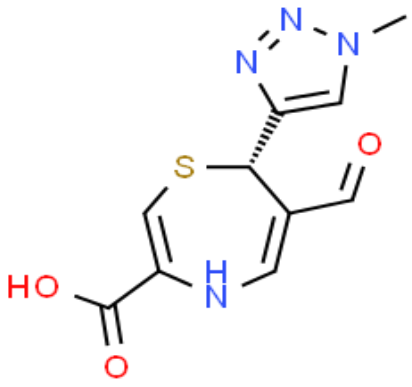

Figure 3: (7r)-6-formyl-7-(1-methyl-1h-1,2,3-triazol-4-yl)-4,7-dihydro-1,4-thiazepine-3 carboxylic acid.

In vitro Antifungal bioassays

The antifungal activities of AcA and its complexes were carried out as reported by Oke *et al.*[24] using already identified fungi isolates from Microbiology laboratory of Bowen University, Nigeria. Fungi used are: *Rhizopus stolonifar*, *Penicillium citrinum, Aspergillus flavus, Aspergillus fumigatus and Aspergillus Niger*.

In silico Antibacterial assays (Molecular Docking)

Two hydrolase enzymes (PDB ID: 1Y54; resolution = 2.10/Å) and (PDB ID: 7LJI; resolution = 1.85/Å) were downloaded.[25] They were prepared using the Protein preparation Wizard in the Maestro/Schrodinger suite.[26-27] The compound AcA, some standard drugs (cefuroxime, tetracycline and streptomycin) and the co-crystallised ligand accompanying the enzyme 1Y54, (7r)-6-formyl-7-(1-methyl-1h-1,2,3-triazol-4-yl)-4,7-dihydro-1,4-thiazepine-3-carboxylic acid (FDT) were prepared using LigPrep at OPLS3 force field level.[28] The site maps were generated[29] before docking of the ligands and the standard drugs in the active sites of the receptors (1Y54: x = 7.49, y = 30.3, z = 25.01; 7LJI: x = -5.62, y = 25.21, z = 52.9) using Glide.[30] The SP (standard precision) and XP (extra precision) docking scores were computed.

Results and Discussion

The FTIR spectrum for AcA (Fig. 5a) showed absorption bands around 3420cm^{-1} and 1299 cm^{-1} indicating an axial ArOH deformation. The –COOH was observed around 1655cm^{-1} and the Ar-H band shows around 3008cm^{-1}, 2924 cm^{-1}and 2893cm^{-1} are indicative of aliphatic CH while 1604cm^{-1} and 1448cm^{-1} were also detected for C=C aliphatic and C=C aromatic, respectively. These results are comparable to that of da Silva *et al.*[14] Comparatively, the absorption bands of the IR spectra of the AcA complexes showed little deviations from that of the AcA as expected due to the complexation process.[31] The following changes were observed in the following bands for both complexes: the hydroxyl group shifted to 3470cm^{-1} and 3550cm^{-1} (for AcA-Cu and AcA-Co) respectively. This variation may be attributed to the coordination of the phenolic grouping of the AcA molecule.[32] The carbonyl group changed to 1620cm^{-1} and 1690cm^{-1} (for AcA-Cu and AcA-Co) respectively (a lower wavelength for AcA-Cu and higher wavelength for AcA-Co) which may indicate coordination of the -COOH group to the central metal (II) ions.[14,31] Variations in the IR absorption bands suggest coordination through the hydroxyl group oxygen atom which is confirmed by the appearance of bands in the region between 437 and 475cm^{-1} as shown in Table 1. Figure 3 shows the proposed structures for the synthesised complexes, where M=Cu^{+2} and Co^{+2}. The data and spectra for IR analyses are shown in Table 1 and Figure 5, respectively.

Ultraviolet Spectroscopy

The Complexes AcA-Co and AcA-Cu in dimethyl sulfoxide (DMSO) gave electron spectra that showed a bathochromic shift in bands I, II and III (Table 2).

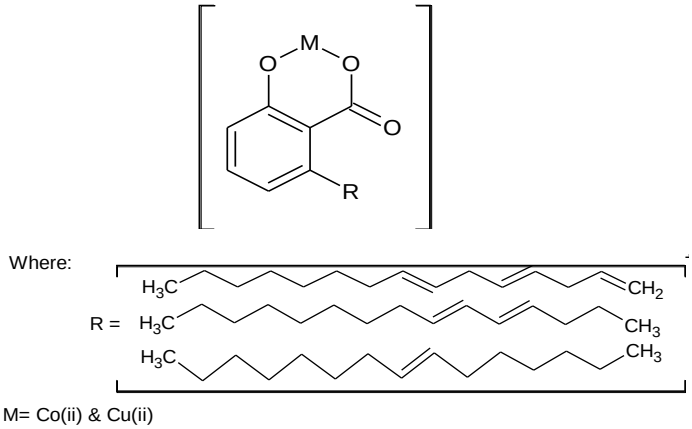

Figure 4: The proposed structure for the complexes with the anacardic acid molecule.[14]

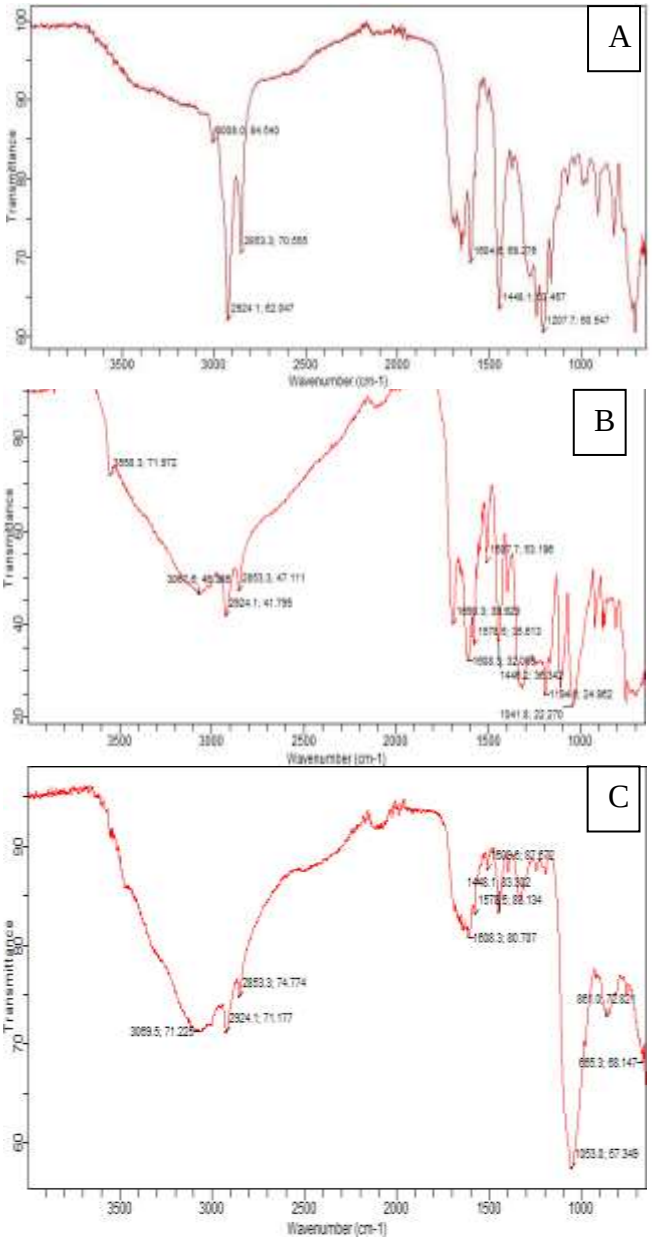

Figure 5: The FTIR spectra of (a) AcA, (b) AcA-Co and (c) AcA-Cu

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Table 1: FTIR spectroscopy data of anacardic acid and the hydrated metal (II) complexes

Compound	ν(O–H) / cm^{-1}	ν(=C–H) ar / cm^{-1}	ν(C=O) / cm^{-1}	ν(C=C) ar / cm^{-1}	ν(C=C) al / cm^{-1}	ν(M–O) / cm^{-1}
AcA	3430	3008	1655	1604	1448	-
AcA-Co	3550	3067	1690	1608	1448	475
AcA-Cu	3470	3069	1620	1608	1448	437

Table 2: Visible spectroscopic data of AcA, AcA-Co and AcA-Cu in DMSO

Compound	Band I / nm	Band II / nm	Band III / nm	Published data	Reference
AcA	306	311	365	246, 314	14
AcA-Co	359	385	542	-	-
AcA-Cu	392	491	528	243, 472	14

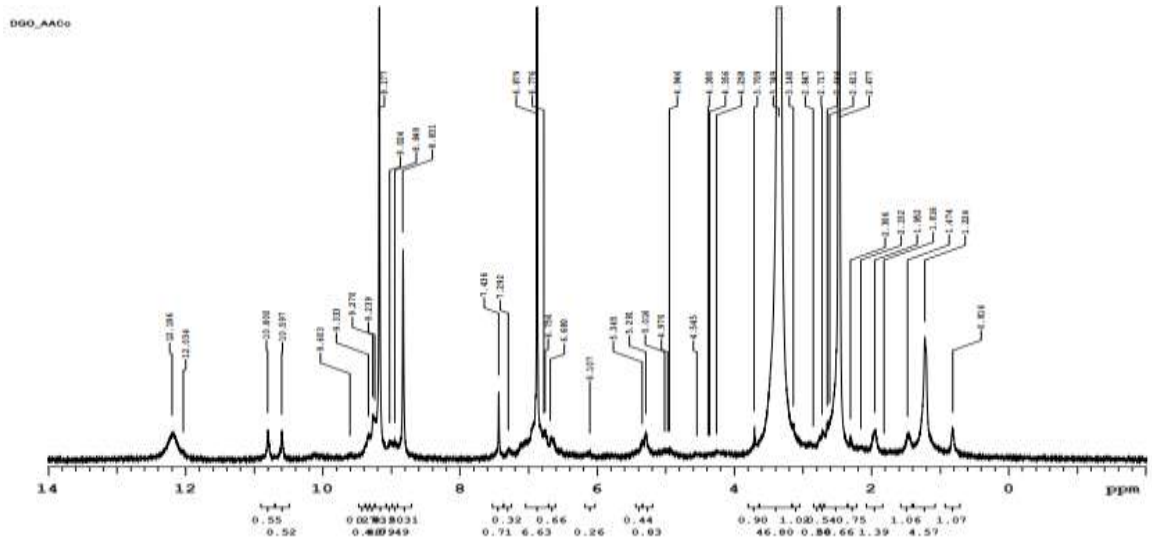

Figure 6: ^{1}H NMR spectrum of AcA-Co

Table 3: NMR spectra data of Anacardic acid

Position	DEPT	δ_H(ppm) (multiplicity, *J* in Hz)	δc(ppm)
1	CH_3	0.82-0.83 (m)	14.32-14.10
2	CH_2	1.23 (s)	22.84-22.50
3	CH	1.27-1.37 (m)	25.58
4	CH	1.47 (s)	27.06
5	CH_3	1.98 (dt, *J* = 13.4, 6.6)	28.81
6	CH_3	2.58 (t, *J* = 7.9)	29.68
7	CH	2.76 (dq, *J* = 18.9, 6.2)	31.63
8	CH_3	3.74 (d, *J* = 2.1)	34.31
9	Ar-OH	4.99 (dd, *J* = 24.2, 13.7)	52.11
10	=CH	5.37 (t, *J* = 4.7)	128.05
11	Ph-H	6.67 (dd, *J* = 15.2, 7.8)	129.30
12	Ph-H	7.01 – 6.76 (m)	129.98
13	Ph-H	7.12 (q, *J* = 10.2)	136.95
14	CH	8.85 (s)	138.46
15		9.04 (d, *J* = 11.1)	138.83
16		9.20 (s)	142.69
17		9.28 (d, *J* = 10.4)	145.79
18	Ph-COOH	12.30 (s)	168.11

These NMR values are similar to that of Morais *et al*.[33]

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This could be because of interaction of the metals with the AcA OH group, "resulting in an electronic redistribution between the free molecules and the metal ions thereby forming an extended ligand system."[14]

[1]H-NMR Spectroscopy

The [1]H-NMR results of anacardic acid shows protons in various chemical environments: the signal at 12.3ppm can be attributed to the –COOH proton for saturated, mono, di and triene AcA. The aromatic signals for these four groups are indicated between 7.12 and 6.67ppm while the signals around 0.89 and 1.98ppm accounts for the protons on the side alkyl groups. AcA-Co has signals different from the above (fig. 6) in that the –COOH proton slightly moved to 12.19 ppm while the aromatic moiety signals are now between 6.88 and 7.44ppm which can be attributed to the presence of the Co^{II} effect which also affects other signals as presented in data in table 3. For the AcA-Cu, there is no significant information for the NMR due to the magnetic properties of copper.

Anacardic Acid

AcA was isolated as a UV-Vis (DMSO) λ / nm 306, 311, 356; FTIR ν / cm-1 3430, 3008, 2924, 2853, 1680, 1604, 1448, 1299, 1207, 1165 and 701. These data are comparable with that of da Silva *et al*.[14]

Anacardic Acid complex of cobalt (II) (AcA-Co)

AcA-Co was synthesized as a black crystalline solid. UV-Vis (DMSO) λ / nm 359, 385, 542; FTIR ν / cm-1 3550, 3067, 2924, 2853, 2100, 1690, 1608, 1578, 1507, 1446, 1194, 1041, 910, 880, 810, 765; IR (KBr) ν / cm-1 735, 475.

Anacardic Acid complex of copper (AcA-Cu)

AcA-Cu was synthesized as a brown crystalline solid. UV-Vis (DMSO) λ / nm 392, 491, 528; FTIR ν / cm-1 3470, 3067, 2924, 2853, 2100, 1608, 1578, 1509, 1448, 1321, 1160, 1194, 1053, 861, 665; IR (KBr) ν / cm-1 613, 437. These data are comparable with that of da Silva *et al.*[14]

In vitro Antifungal Assay

The antifungal assay gave negative results for AcA in all the five fungal strains evaluated, although it has been reported to inhibit *Colletotrichum capsici* spore germination.[34] However; some significant results were obtained with the complexes. Both complexes have their highest activities against *Penicillium citrinum* with AcA-Co having 30±7.07mm and AcA-Cu having 15±2.83mm. None of the complexes showed activity against *Aspergillus flavus* as shown in Table 6. This is an encouraging result that the complexes may give hope in drug discovery. Clotrimazole was the most effective with *Aspergillus fumigatus* (31±2.82mm) but less effective when compared with the complexes against *Penicillium citrinum* (12.5±0.71mm).

In silico Antibacterial assays (Molecular Docking)

AcA showed better docking score when docked with *enterobacter* hydrolase IY54's active sites and is better than cefuroxime, tetracycline, and streptomycin antibacterial standard drugs with binding affinities of -6.164kcal mol^{-1}, -5.036kcal mol^{-1}, -4.345kcal mol^{-1} and -3.820kcal mol^{-1} respectively for SP (Table 7). This trend was repeated for XP except that streptomycin with -7.151kcal mol^{-1} was better that AcA with -5.424kcal mol^{-1}. AcA (−6.164 and -5.424kcal mol^{-1}) showed conventional hydrogen bond with 1Y54 for SP (Ser318 and Tyr150) and XP (Asn346, Ser318, Arg349) docking respectively from its carbonyl and hydroxyl oxygen atoms from the acid group (Figures 8 and 9).

Table 4: [1]H-NMR spectrum data of Anacardic acid complex of cobalt (AcA-Co)

Position	DEPT	δ_H(ppm) (multiplicity, *J* in Hz)
1	Ph-COOH	12.20 (s)
2		10.80 (s)
3		10.60 (s)
4		9.26 (d, *J* =12.1)
5		9.18 (s)
6	CH	8.83 (s)
7	Ph-H	7.44 (s)
8	Ph-H	6.88 (s)
9	=CH	5.29 (s)
10	CH_3	1.96 (s)
11	CH	1.46 (s)
12	CH_2	1.21 (d, *J* = 8.3)
13	CH_3	0.82 (s)

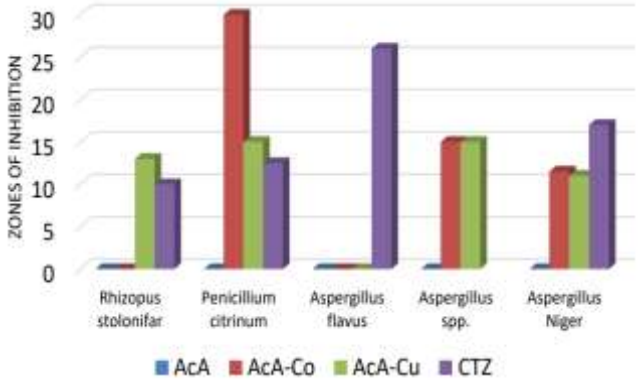
Table 5: [1]H-NMR spectrum data of Anacardic acid complex of copper (AcA-Cu)

Position	DEPT	δ_H(ppm) (multiplicity, *J* in Hz)
1	=CH	5.34 (s)
2	CH	1.99 (s)
3	CH_2	1.20 (s)
4	CH_3	0.81 (s)

Table 6: Antifungal activities of anacardic acid and its hydrated metal II complexes

Fungal strain	Zones of inhibition / mm			
	AcA	AcA-Co	AcA-Cu	CTZ
Rhizopus stolonifar	-	-	13 ± 2.83	10 ± 0
Penicillium citrinum	-	30 ± 7.07	15 ± 2.83	12.5 ± 0.
Aspergillus flavus	-	-	-	26.5 ± 5.
Aspergillus fumigatus	-	15 ± 0	15 ± 0	31 ± 2.8
Aspergillus Niger	-	11.5 ± 3.54	11 ± 1.41	17 ± 2.8

CTZ = Clotrimazole



CTZ = Clotrimazole

Figure 7: Histogram representation of antifungal activities of AcA and its complexes.

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Table 7: Docking result of AcA, FDT and standard drugs with 1Y54

Compound	Binding Affinity / kcal mol^{-1}	Amino Acid	Interactions
***Enterobacter Hydrolase* 1Y54 (SP)**			
Co-ligand	-6.259	ASN 152, ASN 346, SER 64	Hydrogen bonding
AcA	-6.164	SER 318, TYR 150	Hydrogen bonding
Cefuroxime	-5.036	GLN 120, GLU 272, ASN 348, SER 289, TYR 221	Hydrogen bonding, pi-pi stacking
Tetracycline	-4.345	ARG 204, TYR 221, SER 318, SER 64	Hydrogen bonding, salt bridge
Streptomycin	-3.820	SER 343, ASN 346, SER 289, SER 318, THR 316, TYR 150, GLU 272	Hydrogen bonding, salt bridge
***Enterobacter Hydrolase* 1Y54 (SP)**			
Co-ligand	-4.641	ASN 346, SER 318, LYS 67, SER 64, ASN 152	Hydrogen bonding, salt bridge
AcA	-5.424	ASN 346, SER 318, ARG 349	Hydrogen bonding, salt bridge
Cefuroxime	-3.660	GLU 272, THR 316, LYS 315, TYR 150, ASN 346	Hydrogen bonding, salt bridge
Tetracycline	-2.534	SER 64, SER 318, TYR 221, ARG 204, ASN 346	Hydrogen bonding, pi-cation
Streptomycin	-7.151	GLN 120, SER 64, SER 318, ARG 204, SER 343	Hydrogen bonding

1Y54 (2.10)- *Hydrolase enterobacter* (x = 7.49, y = 30.3, z = 25.01)

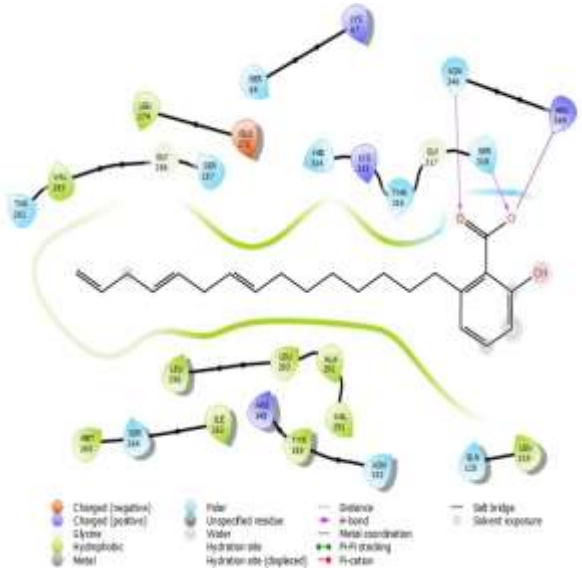

Figure 8: AcA with 1Y54 XP

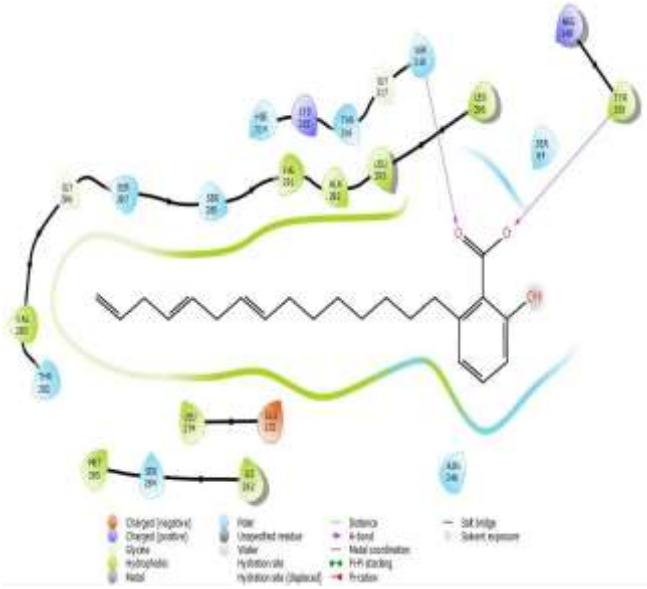

Figure 9: AcA with 1Y54 SP

Conclusion

Anacardic acid was isolated from the liquid derived from cashew nut shell and used to synthesised Co^{II} and Cu^{II} complexes. AcA complexes gave good results against different fungi used. The antifungal activity of the complexes against *Penicillium citrinum* were significant; giving better results than clotrimazole; which was used as the standard drug. The *in silico* antibacterial evaluation of AcA with *Enterobacter Hydrolase* IY54 SP and 1Y54XP also showed better interactions with proteins compared to tetracycline, streptomycin and cefuroxime which are established antibacterial standard drugs. With these results from AcA and its complexes, further studies are recommended to ascertain and establish them as better antifungal and antibacterial drugs.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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