Journal of Advances in Microbiology Research

E-ISSN: 2709-944X
P-ISSN: 2709-9431
JRM 2023; 4(2): 25-32

www.microbiojournal.com
Received: 29-04-2023
Accepted: 03-06-2023

Ocheni GR
Department of Biosciences, College of Natural and Applied Sciences, Salem University, Lokoja, Kogi State, Nigeria

Omowaye OS
Department of Biosciences, College of Natural and Applied Sciences, Salem University, Lokoja, Kogi State, Nigeria

Makolo D
Department of Microbiology, Faculty of computing and Applied Sciences, Baze University, Abuja, Nigeria

Emmanuel SE
Department of Biosciences, College of Natural and Applied Sciences, Salem University, Lokoja, Kogi State, Nigeria

Correspondence Author;
Ocheni GR
Department of Biosciences, College of Natural and Applied Sciences, Salem University, Lokoja, Kogi State, Nigeria

In silico and *in vitro* antimicrobial testing of ethanol extract of *Xylopia aethiopica* against *Salmonella typhimurium* and *Shigella* species isolated from Wistar rats infected with *Trypanosoma evansi* and *Trypanosoma brucei brucei*

Ocheni GR, Omowaye OS, Makolo D and Emmanuel SE

DOI: https://doi.org/10.22271/micro.2023.v4.i2a.95

Abstract
Plants with antimicrobial potential have been implicated in the treatment of infections. Studies have shown that extracts from diverse plants have shown antibacterial and or antitrypanosomal activities against infectious agents. This research examined the *in vitro* antimicrobial testing of ethanol extract of *Xylopia aethiopica* against *Salmonella typhimurium* and *Shigella* species isolated from wistar mouse already infected with *Trypanosoma evansi* and *Trypanosoma brucei brucei*. Sixty (60) wistar mouse of 16 weeks old were used and were randomly grouped into 8 groups where N = 5 in group A, B, E, F,G and H. N=15, in C and D. group A: control, group B uninfected but treated (346.4 mg/kg/bwt XAEE. Group C infected untreated (*T. Brucei* brucei), Group D infected untreated (*T. evansi*). Group E infected treated (*T. Brucei* brucei 346.4 mg/kg/bwt XAEE). Group F. infected treated (*T. evansi* 346.4mg/kg/bwt XAEE). When molecular docking simulation was used, lots of phytochemicals were identified and screened in the XAEE used in this study. Findings from this study have shown an indication of *in vitro* antimicrobial activities of ethanol extract of *Xylopia aethiopica* fruits against *Salmonella typhimurium* and *Shigella* species examined.

Keywords: Antimicrobial activity, *Xylopia aethiopica*, Negro pepper, *Salmonella typhimurium*, *Shigella*, molecular simulation

1. Introduction
Plants has been used in the treatment of human common infectious diseases since ancients times and some of these traditional medicines are still included as part of the habitual treatment of various maladies such as bacteria, fungi, parasites and viral diseases (Kafaru, 1994; Emmanuel *et al*., 2021) [32, 8].

Sleeping sickness, also known as human African trypanosomiasis, is a vector borne parasite illness. The implicated parasites are protozoa from the genus Trypanosome. These are spread to people via the bites of tsetse flies (Genus *Glossina*), which became infected after feeding on animals that harboured human pathogenic parasites (David *et al.*, 2013) [5]. The sickness primarily affects inhabitants in Africa's isolated rural areas, and it is lethal if left untreated. Travellers who pass through areas where the vector is common run into the risk of contracting the disease. Although rare instances have been reported in the suburbs of big cities in several disease-endemic nations, the disease is generally not found in urban settings. According to Bala (2009) [4] only 36 of the sub-Saharan African nations have tsetse flies that can spread sleeping sickness. A World Health Organization (WHO) expert committee estimated in 1995 that there were 60 million people at risk and 300,000 new cases were being identified and treated each year. Humans are infected by two types of trypanosomes called *Brucei brucei*, specifically *T. Brucei gambiense and T. Brucei rehodesiense*. Over 90% of cases that have been reported are caused by *T. Brucei gambiense*, which also causes chronic illnesses that might take weeks, months, or years before symptoms appear and have a three-year life span. On the other hand, *T. Brucei rehodesiense* causes an acute form of infection and can cause fatality within weeks; it is more virulent and rapidly developing than *T. Brucei gambiense* (Kabiru 2013) [16]. Since chemotherapies and vector control systems have been the mainstays of the disease fight, their efficacy and safety continue to be a source of worry due to drug side effects and parasite drug resistance.

Due to this conundrum, efforts to find novel chemical compounds for the treatment of human African trypanosomiasis (HAT) must continue. This work is intended to screen this medicinal plant, *Xylopia aethiopica*, in Kogi State, Nigeria. This plant has been used traditionally by traditional medicine practitioner (Ezekwesili *et al*, 2010) [2].

A medicinal plant with a pleasant scent called *Xylopia aethiopica* is usually found in Africa's lowland rain forests or its extreme woodland savannah zones. This plant belongs to the amnonacease family and can grow up to 20 meters tall (Karawya *et al*., 1979) [33] Almost all of *X. aethopica's* morphological components, including the leaf, stem, root, bark, and fruit (both dry and fresh), are used in traditional medicine to cure a variety of illnesses, including rheumatism, headache, malaria, asthma, and dysentery, among others.

2. Methodology

2.1 Collection and Identification of plants

Xylopia aethiopica fruits were bought from Lokoja International Market, Kogi State of Nigeria in the month of August, 2022. Authentication of the sample was done in the Microbiology Department of Salem University, Lokoja, Kogi State.

2.2 Plant Extract Preparation

The plant fruit (*Xylopia aethiopica*) was washed in a running tap water and air dried. The air dried sample was then grind into powder with the aid of mortar and pestle. The *Xylopia aethiopica* residue was weighed using a Camry automatic scale (ek3250 A and D Company Limited, Japan) into a dirt free waterless flat bottom flask. Fifty gram (50 g) of the pulverize sample was cold macerated in 500 ml distil water (1:10 w/v), for 48 hours with constant rocking on electronic shaker with model no. 001. The ethanol extract was weighed the same grams, the same procedures was carried out as the aqueous extract as mentioned above. The filtration of both the aqueous and ethanol extract was done by using what man no 1 filter paper, a pore size of 100 (195 mm by 195 mm). The filtrates were concentrated using a rotary evaporator and placed in a water bath at 60" ℃ to allowed evaporation of the solvent as described by (AOAC 2010). The obtained jelly like extract was stored in a sterile Petri-dish in the refrigerator at 4 °C until required for use.

2.3 Phytochemical screening

Xylopia aethiopica extract was qualitatively screened for the presence of Tannis, Alkaloids, Flavonoids, Saponins, Glycosides, fat and oil, Terpenoids, Phytosterole, Phenols, Coumarin as mentioned by Sofowora 1993 [34].

2.4 Microorganisms Tested

T. Brucei brucei and *T. evansi* were obtained from an infected mice bought from the Nigeria Institute of Trypanosomiasis Research (NITR), Jos, Plateau State, Nigeria in the month of August 2022. The parasites were kept in the University animal's house by serial passage in mice until required for use. Passage was carried out when parasitaemia was in the range of 16-32 parasites per field (usually 3-days post infection). In passaging, 1X 10^3 parasites in 0.1-0.2 ml blood/PBS were infected intraperitioneally into normal mice, acclimatized under laboratory condition for two weeks.

2.5 Experimental animals

Sixty (60) healthy wistar mice of sixteen (16) weeks old were bought from the animal house of Salem University Lokoja, Kogi State, Nigeria. They were acclimatized and maintained under 12 hours (light/dark) cycle where they were allowed free access to a pellet diet and clean water aid libtum. Procedures applied with the guide for care and use of laboratory animals following good laboratory practice (GLP) regulations of the World Health Organization (WHO). The laboratory principles for animals care were followed dully.

2.6.1 Plant Extract Sterility

The extract sterility was confirmed using the standard laboratory procedure. *Xylopia aethopica* fruit extract was incubated on sterile nutrient agar and incubated at 37 ℃ for 24 hours. Absence of microbial growth on the extract after incubation proved the extract sterility.

2.6.2 Culture Media Preparation

The culture media used in this research included Salmonella *Shigella* agar (SSA), Nutrient agar (NA), Muller Hinton agar (MHA). All media used were prepared according to the manufacturers' instructions following standard microbiological procedures as described by Sandle, (2017) [25] and Emmanuel *et al*. (2021) [8].

2.7 Experimental Design

Exactly 60 wistar mice of 16 weeks old were chosen randomly into 8 groups with five (5) mice each in group A, B, E, F, G and H, while fifteen (15) each in group C and D. Group A: uninfected un-treated (control), group B un-infected but treated (346.4 mg/kg bwt XAEE), group C infected untreated (*T. Brucei brucei*), group D infected untreated (*T. evansi*), group E infected treated (*T. Brucei brucei)* 346.4 mg/kg bwt XAEE), group F infected treated (*T. evansi*), (346.4 mg/kgbwt XAEE).

Infection of the experimental mice was carried out by injecting 0.1ml of blood containing approximately $1x10^3$ trypanosomes intraperitioneally into each mice in the infected groups. The experiment lasted for 14 days.

2.8 Bacteria Isolates

Pure culture of *Salmonella typhimurium* and *Shigella* species were isolated from the experimental animals by standard bacteriological procedures. Sterile swab stick and wire loop were used to collect samples from the feaces of each test animals. Presumptive Salmonella and *Shigella* sample (from the feaces) was inoculated using a sterile wire loop into the agar plates and inoculated at 37 ℃ for 24 hours.

2.8.1 Authentication of the Isolates

Morphological identification and biochemical characterization were used to authenticate the isolates using the method described by Patel, (2017) [24] and Doaa, (2012) [6].

2.9 Anti-bacteria Susceptibility Test of (XAEE)

The method of Makolo *et al*., 2019 [18] was used in carrying out anti-bacteria susceptibility test using the standard disc diffusion technique following the guidelines of Clinical and Laboratory Standard Institute. Briefly, sensitivity disc were made of Whiteman no 1 filter paper (6mm in diameter). The

sterile disc were impregnated with different concentrations of the ethanol extract (10 mg/ml to 50 mg/ml) were placed aseptically on Mueller Hinton agar that had earlier been inoculated with the test organism. A set of gram negative and gram positive control disc using same agar seeded with the test organisms was set up. All plates (Test and control) were inoculated overnight at 37 ℃ for about 24 hours. The zones of inhibitions were measured according to the method of Jan, 2009, values were recorded in mm.

3. Data analysis

Data were expressed as Mean ± SEM Tabulation of information was employed with data obtained from samples. Frequency distribution percentages and bar charts were used to treat the formulated research questions. While descriptive and inferential statistics (Regression analysis) was used to test the relationship between the variables and the effect of the independent on the dependent variables.

4. Preparation of protein structure of Topoisomerase iv in *Salmonella typhimurim*

The crystal protein structure of topoisomerase iv of *Salmonella typhimurium* was retrieved from the protein Data bank (PDBID: 3FW5). From the retrieved structure, the native ligands were extracted and water molecules were removed. Hydrogen atoms were added to the structures using Auto dock version 42 program Scipps Research Institute. La Julla CA).

4.1 Preparation of Ligand structure of XAEE

The 3D structure of compounds derived from the plant and reference compounds were retrieved from the pubchem data base (www.pubchem.Ncbi.nih.gov) in the structure data formula (SDF). The SDF structures of these compounds and reference compounds were converted to mol-2 chemical format using open Babel. The polar hydrogen charges of the Gasteiger-type were assigned. To atoms in the chemicals and the non-polar molecules of hydrogen were merged with the carbons. The internal degrees of freedom and visions were set to zero. The structures were then converted to the dockable PDBQT format utilizing Auto Dock tools

4.2 Molecular Docking Simulations of XAEE against Topoisomerase IV of *Salmonella*

The ligand structures were imported into Auto Dock vina in PyRx 0.8 (Trott and Olson. 2010) [30] and minimized using the incorporated open Babel by applying the universal force field (UFF) as the energy minimization parameter and conjugate gradient descent as the optimization algorithm. The ligand structures were then screened against the active site of topoisomerase iv. The active site of the protein was defined by the grid boxes. The molecular docking simulations were then analyzed keeping all other parameters as default. After docking simulation, the molecular interactions between the ligands and proteins were viewed and discovery studio visualizer version 16.

4.3 Molecular Dynamic Simulation

The apoenyzmes (PDBID), and the lead compounds complexed with the protein were subjected to full atomistic Molecular Dynamic MD) simulation using GROMACS, 2019; 2 and GROMOS 96 43 al force field on the web GRO server. The ligands topolpgy files were generated using the PRODRG web server (http://davapd. Bioch. Dundee. Ac.Uk/cgi-bin/prodrg). The enzymes and ligands–enzyme complex systems were solvated within a cubic box of the transferable intermolecular potential with a four-point (TIP$P) water model applying the periodic boundary conditions at a physiological concentration of 0.154 M. Set by neutralized salt ions. The minimization of the systems was performed for 10000 steps using the steepest descent algorithm constant number of atoms, volume and temperature ensemble (NVT) embles for 0.3 nanoseconds, followed by 0.3 nanoseconds equilibrium in constant atom number, constant pressure and constant temperature (NPT). The temperature was maintained using 310 k using velocity rescale, while pressure was set to 1 atom using parimello-Rahmanbarostat. Leap. Frog integrator was used with a time step of 2 femto seconds. For each system 100ns of the production run was performed and for every 0.1ns of, a snapshot was saved with a total of 1000 frames from each system. The trajectories were analyzed using VMDTK console scripts to calculate RMSD, RMSF, SASA, RoG, and the number of H-bond, Amino acid interactions of selected phytocompounds with Topoisomerase iv of salmonella were also stimulated.

5. Ethnical Approval

The Nigeria institute of Trypanosomiasis Research (NITR), Jos Plateau State Nigeria authorized the field work.

6. Results

Zone of inhibition produced by XAEE on *Trypanosoma brucei brucei* as shown in Fig. 1 represent the zones of inhibition produced by XAEE on *Trypanosoma brucei brucei*. The ethanol extract of the XAEE exhibited

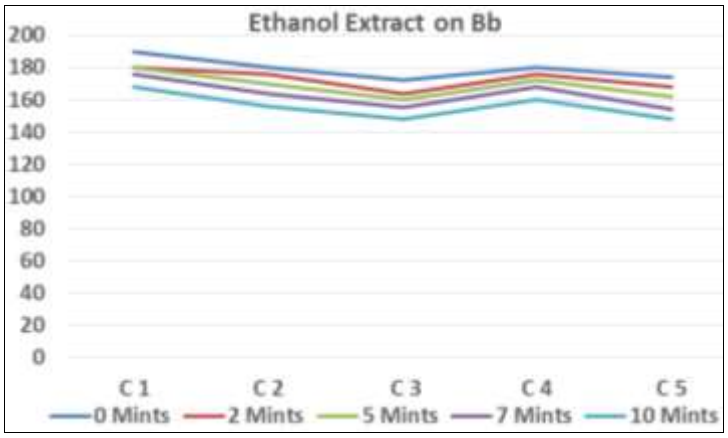

Fig 1: *In vitro* anti-trypanosomes effect of XAEE on *Trypanosoma Brucei brucei* at time intervals

6.1 Binding Affinity of *Xylopia aethiopica* compounds with Topoisomerase IV of *Salmonella*

The binding affinities from the docking analysis of the protein topoisomerase iv (3FV5) for the phytocompounds used against *Salmonella typhimurium* is shown in Table 1. Based on the minimum binding energies and interactions with catalytic residues, the top five phytocompounds with binding energies ranging from (5.5-6.6) Kcal/mol) were compared with the binding energies of the reference compounds 1- (acetyl-6pyridin 3-yl-1 h-benzinidazole-2-yl) -3ethyl urea (PBE) (-6-/kcal/mol). From the interaction of the top phytocompounds with topoisomerase iv, three compounds with the highest binding energies compared to the reference compounds were selected, Clorobiocin kavr-15-ene, Kavr-16-ene, two phytocompounds that had highest binding energies to that of the reference compounds were selected. Heptodecanole, Triamcinolone Acetonide as shown in Table 1 and 2.

Table 1: Binding energy score for the interactions between selected screen phytocompounds in XAEE and topoisomerase iv of *Salmonella typhimurium*

S/N	Phytocompounds	DNA gyrase (1tm2)
1	Chlorobiocin	-6.6
2	Kaur-15-ene	-6.1
3	Kaur-16-ene	-6.1
4	Heptadecanolide	-5.7
5	Triamcinolone Acetonide	-5.5
6	4-Dibenzofuranamine	-5.4
7	Carveol	-5.3
8	2-Dibenzofuranamine	-5.2
9	Columbin	-5.2
10	(-)-Spathulenol	-5.1
11	2, 6-Dimethyl-4-phenylpyridine	-5.1
12	Fumaric acid, 2, 4-dichlorophenyl hexyl ester	-5.0
13	Thymol	-5.0
14	Anethol	-4.8
15	Cis-5, 8, 11, 14, 17-Eicosapentaenoic acid	-4.8
16	Linoleic acid	-4.8

Table 2: Interaction between top ligands and topoisomerase iv of *Salmonella typhimurium*

Compounds	Hydrogen bonds interactions		Hydrophobic interactions	
	Number	Residues	Number	Residues
Chlorobiocin	8	Gly 38, Tyr 62 (4), Gln 59, Thr 61, Thr 43,	4	Tyr 62(2), Lys 54, Val 60
Kaur-15-ene	0	Nil	4	Val 269(3), Tyr 338
Kaur-16-ene	0	Nil	4	Val 269(3), Tyr 338
Heptadecanolide	1	Asp339	1	Val 269
Triamcinolone Acetonide	2	Thr61,	3	Val 60, Arg 29, Tyr 85

Amino Acid Interaction of Selected Phytocompounds with Topoisomerase IV *Salmonella typhimurium*

The amino acid interaction of topoisomerase iv with reference compounds and five ranked phytocompounds that demonstrate the highest binding tendencies are represented in fig. 2-6 showing the 2D and 3D structure. The interaction of the protein residues with individual ligands groups were majorly through H-bond, hydrophobic interactions and few other bonds (Table 2 of the supplementary file). In the Chlorobiocin- 3FV5 complex, a conventional hydrogen bond and carbon hydrogen bond were formed with Gly38, Tyr 62(4), Gin59, Thr61, Thr43 respectively while the alkyl interaction was formed with Tyr62 (20, lys54, Val60 (fig.12). In the Kaur-15-ene 3FV5 complex a conventional hydrogen bond were not formed while the alkyl interaction was formed with Val269 (3), Tyr338 fig.13). In the Kaur-16-ene 3FV5 complex, a conventional hydrogen bond and carbon (Heptodecanolde 3FV5 complex conventional hydrogen bonds were formed ASP339, while the alkyl interaction was formed Val269 (fig.!5). In the Triamcinolone acetonide 3FV5 complex a conventional hydrogen bonds was formed with Thr61 while the alkyl interaction was formed with Val.60, Arg.20, Tyr 85.

7. Discussion

Test involving susceptibility is used to evaluate the resistance or sensitivity of pathogenic aerobic and facultative anaerobic bacteria to several antimicrobial phytocompounds or compounds to help in the drug therapeutic selection option. (Jan. 2009) [14]. In this study, *Salmonella Typhimurium* was observed not be sensitive to all the concentration of the *Xylopia aethiopica* ethanolic extract (XAEE). Gram negative bacteria were more resistant to the *Xylopia aethiopica* extract than the gram positive bacteria. This report was in agreement with that of (Okigbo *et al*, 2015) [22], and (Nwinyi *et al.*, 2009) [21], who reported that gram negative bacteria are more resistant to anti-bacteria agents than the gram positive species. This may be due to the difference in the cell wall composition and structure, especially the polysaccharide and protein outer membrane in the cell wall of the gram negative bacteria that reduce diffusion of antimicrobial agents into the cell.

Chea *et al*., (2021), have attributed the difference in the antibacterial effect between gram- positive and gram-negative organisms to the structural composition of the bacteria cell. Gram-positive bacteria (*S. aureus*) have a relatively broad peptidoglycan layer that is fully permeable to substances, thus making it more sensitive to extract. Gram- negative bacteria (*S. typhimuriun*) have thick lipopolysaccharide bilayers embedded with carrier proteins whose channel size determines the size of molecules to pass through. The larger size of the plant bioactive compounds

may not be able to pass through into the bacterial cell, thus being less sensitive to extracts.

Parallel advances in protein crystallography and various virtual screening software for the modeling of ligand-receptor interactions have enhanced computer- aided drug designed. In this this study, a structure- based virtual screening of phytocompounds was employed via a competitive docking approach for topoisomerase iv agonist with a dual inhibitory potential against *S. typhimurium*.

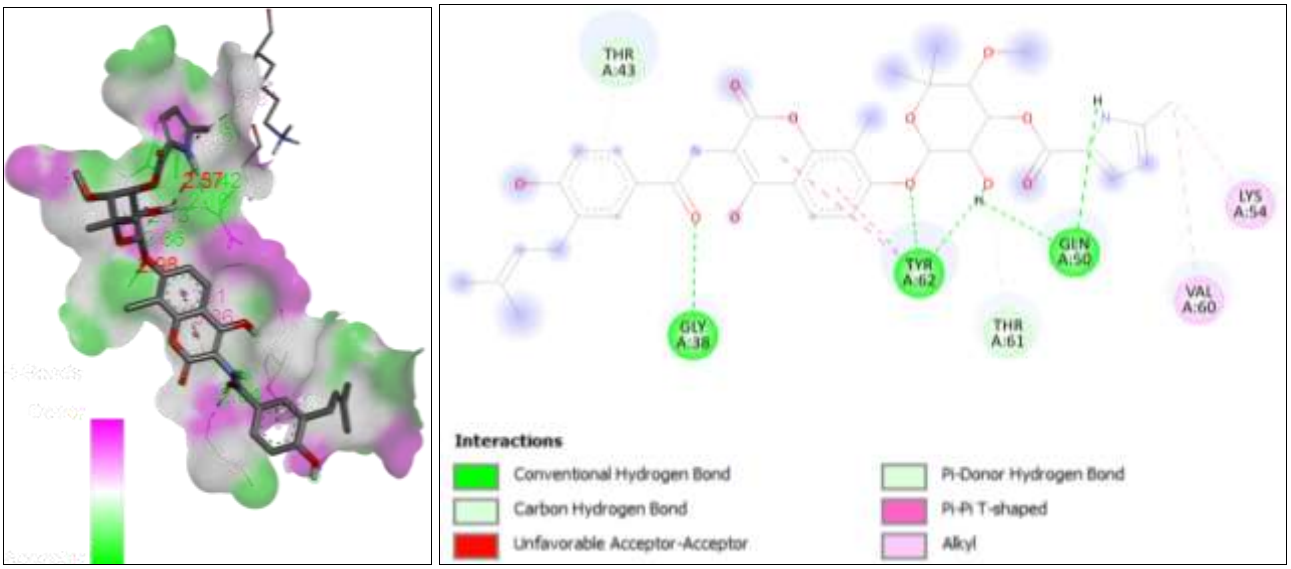

Fig 2: The structure Chlorobiocin complex

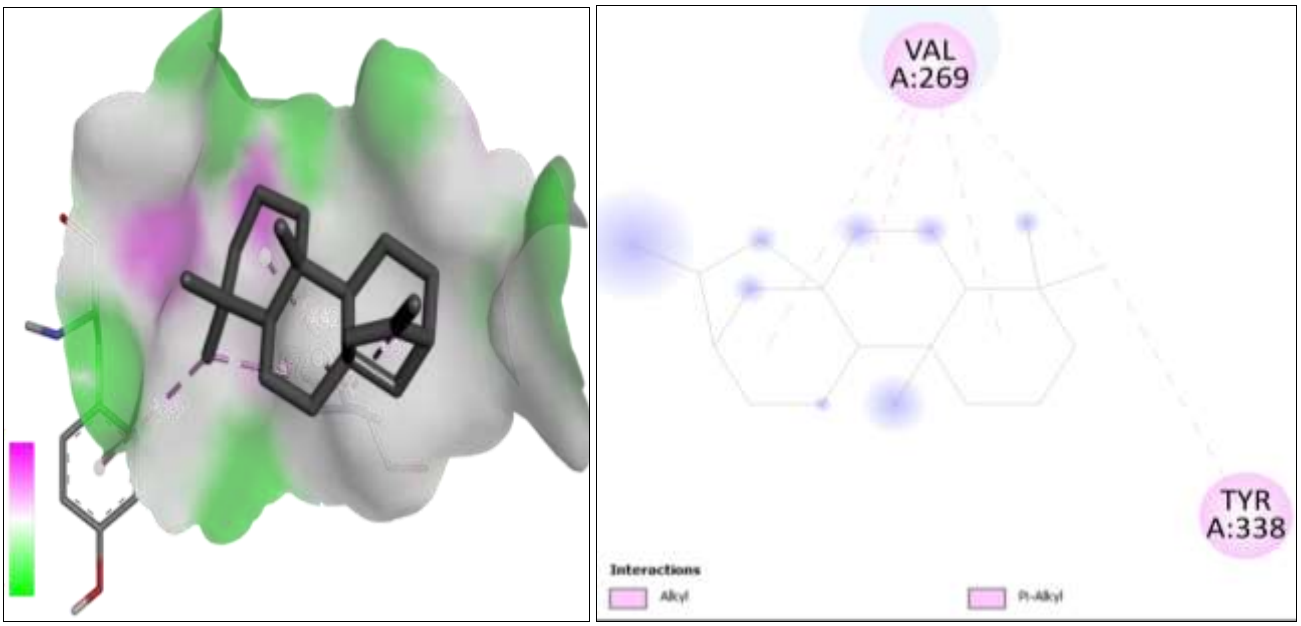

Fig 3: The structure of Kaurene-15 ene complex

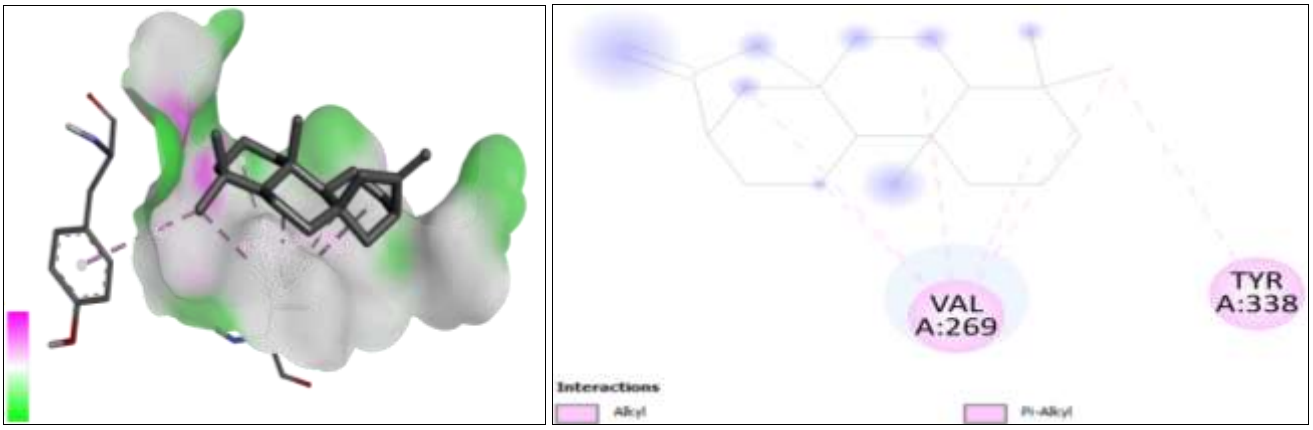

Fig 4: The structure of Kaurene-16 ene complex

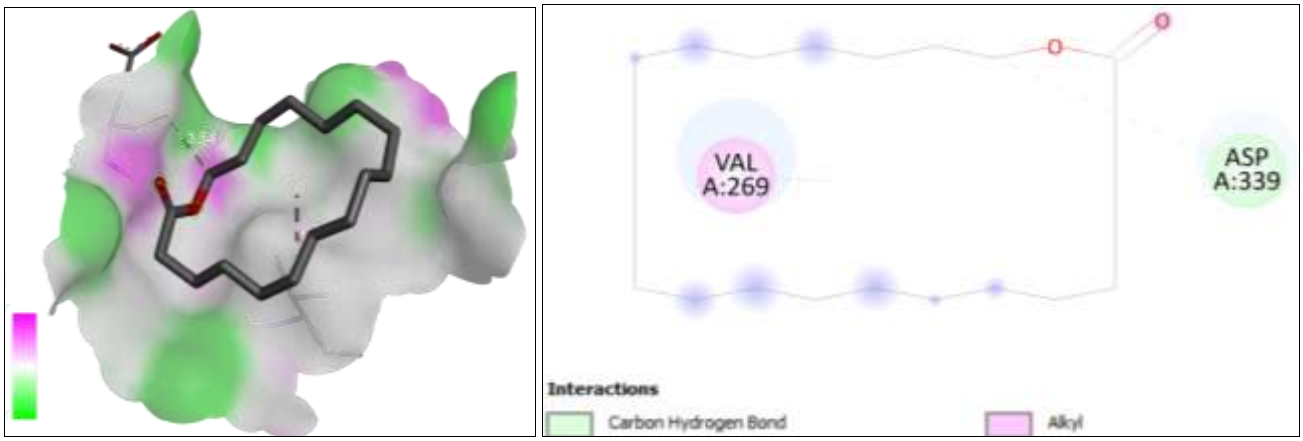

Fig 5: The structure of Heptadecanolide complex

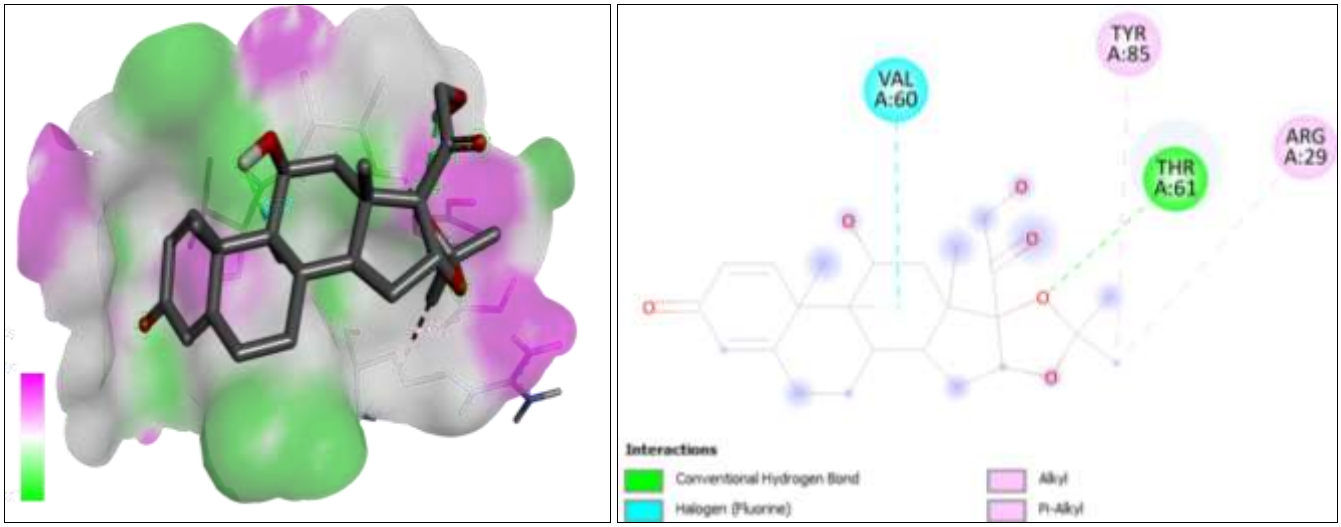

Fig 6: S. The structure of Triamcinolone Acetonide complex

The top five phytocompounds for topoispmeraseiv were further analysed for anti-bacteria effect and they were competitively and selectively docked. They were docked into hydrophobic ligands bing pocket (9LBP) which is located in the bottom half of the GR ligand binding domain, (LBD) (Morris *et al*., 2009) [35]. The top five compounds were Chlorobiocin, Kaur-15-ene, Kauur-16-ene, Heptadecanolide and Triamciinolone Acetonide (-6.6, -6.1, -6.1, -5.7, -5.5 Kcal/mol) and the reference compounds (PBE) had -6.1Kcal/mol. These phytocopounds present might be responsible for the pharmacological property of *Xylopia aethiopica*, exerting the antimicrobial effects, as it is relative of (Nnodin *et al*, 2011).

8. Conclusion

This study established the *in vitro* antimicrobial activity of ethanol extract of *Xylopia aethiopica* against *Salmonella*. The results gotten from the study showed Chlorobiocin phytocompounds (Chlorobiocin, Kaur-15-ene, Kaur-16-ene, Heptadecanoolide and Triamcinolone acetonide) obtained from *Xylopia aethiopica* showed effective interactions with the topoisomerase iv of *Salmonella*, making the plant promising enough exert an antimicrobial effect. This study thus has shown that *Xylopia aethiopica* (Neggro pepper) could have antimicrobial potential and as such be considered a formidable source for the search for new drugs against bacteria, hence the need for further research on better methods of extraction such as activity- guided fractionation of the crude extract to establish and concentrate the fraction where the bioactivity against bacteria lies and the degree of the antimicrobial potential of *Xylopia aethiopica*.

Conflict of interest

The authors declare no conflict of interest

Acknowledgements

The Department of Microbiology, College of Natural and Applied Science, Salem University, Lokoja Nigeria, is acknowledged for providing the basic equipment and infrastructure for carrying out the research work

Authors Contribution

Ocheni Gloria Ramotu: Methodology, Investigation, Resource Project administration, Writing-original draft.

Olaniyi Stephen Omowaye: Conceptualization, Methodology, Analysis, Investigation, Resource, Supervision, Project administration, Writing-review and editing.

Daniel Makolo: Methodology, Investigation, Resource, Supervision, project administration, Writing-review and editing.

Emmanuel Sylvester Eneojo: Methodology, Investigation,

Resource, Project administration, Writing-review and editing

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How to Cite This Article
Ocheni GR, Omowaye OS, Makolo D, Emmanuel SE. *In silico* and *in vitro* antimicrobial testing of ethanol extract of *Xylopia aethiopica* against *Salmonella typhimurium* and *Shigella* species isolated from wistar rats infected with *Trypanosoma evansi* and *Trypanosoma brucei brucei*. Journal of Advances in Microbiology Research. 2023;4(2):25-32.