Antibacterial Aanalysis of WWithanolides from *Datura innoxia*

AbstractABSTRACT

Objectives: This study was aimed to explore the therapeutic potentiality of *Datura innoxia* through the chemoinformatic and antibacterial evaluation of withanolides extracted from it.

Methodologys: The pPharmacokinetic and pharmacodynamic properties and drug-likeness of the withanolides ; wWithametelinol A, wWithametelinol B, wWitharifeen, wWithametelin, dDinoxin B, and dDaturacin of *D. innoxia* were analyzed using the SwissADME program. Schrodinger software was used to target and evaluate their antibacterial potentialities through docking studies. The pPenicillin-binding protein, DNA gyrase, eEfflux pump protein, and qQuorum sensing regulators of *S. aureus* and *E. coli* were selected as target proteins for assessing protein–ligand interaction. All observations were comparatively analyzed with the properties of withanolide A and withaferin A, the best-known withanolides. Most active dDinoxin B withanolide (12500–100000 µg/ml) extracted from leaves of *Datura innoxia* and confirmed through LC-ESI-MS; is subjected to antibacterial assay following the agar diffusion and macro broth dilution methods against mMethicillin-rResistant *S. aureus* (MRSA) and mMulti-dDrug resistant *E. coli* isolated from the urine samples of patients.

Results: *In-silico* studies revealed the therapeutical properties of various withanolides present in *D. innoxia*. EsIn pecialrticulyar, the drug-likeness and antibacterial properties of withametelin and dDinoxin B wereare significantly and remarkably high due and remarkable due to their binding affinity towards cell membrane proteins. Docking studies have shown the efflux pump protein of *E. coli* and penicillin-binding proteins of *S. aureus* to beas the target. A sThe Ssignificant antibacterial assay revealed that the MRSA isolates were susceptible to dDinoxin B, with a zone of inhibition of 21±0.5 mm to 24±0.5 mm, and the bacteria were susceptible at a concentration rate of ≤ 12.5 mg/ml.

Conclusion: It is crucial to bring It is to bring awareness of the therapeutical importance of *D. innoxia* and to preserve this vital plant from gbetting massively largely destroyed. As computational studies promote the effective selection of drug molecules, this research also helps to select the best compound for further clinical analysis.

Keywords:
Datura innoxia; wWithanolides; cChemoinformatic evaluation; mMethicillin-rResistant S. aureus; mMulti-dDrug resistant E. coli.

1. INTRODUCTIONntroduction

Withanolides have attracted the scientific community''s interest in recent years, due to their structural properties and demonstration of considerable pharmacological effects, such as anti-inflammatory, antitumor, immunomodulatory, and antimicrobial effects [1]. Approximatelyanti-inflammatory, antitumor, immunomodulatory and antimicrobial[1]. Approximately 750 withanolides with more than twenty-two carbon skeletons have beenare reported from various plant sources. In the Solanaceae, family, withanolides are present in twenty-five genera [2]. Even thenOf themse, Withaniatwenty-five genera[2]. Even then Withania and Physalis have been selected most extensively for their therapeutical analysis. Nearly 130 withanolides have been were extracted from various parts of Withania somnifera, a traditional Ayurvedic plant. This plant has the highest known number of withanolides of any species so far, and withanolide A and withaferin A have been found bareing thto be thee best antibacterial withanolides found in it [3].in it[3].

AsIn exploring studies on withanolides, the presentis research highlights unspoken unstudied Datura species with itand their identified withanolides. Despite its reputation as a harmful plant due to its poisonous components, it can be purified to produce medically beneficial compounds [4]. Thebeneficial

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~~compounds[4]. The~~ presence of withanolides is seen in many species of this genus, such as *D. metel* [5]. *D.as D.metel[5]. D. innoxia* [6], *D. stramonium* [7], *D. wrightii* [8], and *D. ferox* [9].

D. innoxia (Fig. 1) is native to the American Southwest, Mexico, and Central America, as far south as Belize and Guatemala, but today is~~so~~ common in ~~Asian~~ tropical Asian regions. *D. innoxia*~~It~~ is a shrubby perennial that grows to a height of 2–5~~2–5~~ feet. Small, silky greay hairs cover the plant's stems and leaves, giving it a graeyish appearance. It has an entire-edged ovate to elliptic leaves. The flowers are ten toothed and white, with a length of 12–19 cm. The plants~~y~~ grow upright at first, then incline downward, ~~and. It~~ blooms~~s~~ from early summer to late autumn. The fruit is an egg-shaped spiny capsule with a diameter of around 5 cm. Atropine, scopolamine, hyoscyamine, withanolides (lactones), and other tropanes are among the active factors ~~in of~~ *D. innoxia*.

Fig. 1. *D. innoxia* in its natural habitat. View from Amity Campus premises, Lucknow, India.

~~Even though~~Although the genus *Withania* is well-known for withanolide compounds, our observation of significant broad-spectrum antibacterial properties ~~the~~ of d~~D~~inoxin B~~b~~ withanolide [10] from *D. innoxia* ~~prompted~~~~b withanolide[10] from *D.innoxia* prompted~~ us to compare the drug likeness and antibacterial properties of different withanolides obtained exclusively from *D. innoxia*~~,~~ using *in-silico* methods. This was undertaken~~It is~~ to bring awareness to~~of~~ the therapeutical importance of this species and to preserve this plant from ~~g~~bet~~t~~ing ~~massively~~ destroyed on a large scale. As computational studies promote the effective selection of drug molecules, this research also helps to select the best compound for further clinical analysis.

~~R~~A review of the literature, as well as Pubchem data [11], showed~~s~~ that w~~W~~ithametelinol A [12], w~~W~~ithametelinol B [12], w~~W~~itharifeen [13], w~~W~~ithametelin [14], d~~D~~inoxin B [10], and d~~D~~aturacin [15] are~~Pubchem data[11] shows Withametelinol A[12], Withametelinol B[12], Withafeen[13], Withametelin[14], Dinoxin B[10], and Daturacin[15], are~~ the identified withanolides ~~from~~ of *D. innoxia* (Fig. 2). In this work, we have evaluated the inhibiting activity of these withanolides with selected target proteins of *S. aureus* and *E. coli* through docking studies, as doing so~~it~~ provides a rational new approach to study the antibacterial properties of drugs. Furthermore, ~~an~~ evaluations of the pharmacological ~~evaluation was also performed, including the evaluation of the pharmacological~~ properties, as per Lipinski's rule of five, drug likeness, bioactivity, and ~~the~~ drug score were all performed. The p~~P~~harmacokinetic~~s~~ properties were evaluated to analyze the interaction of the individual from the time of administration to absorption, distribution, metabolism, excretion ~~as well~~, a~~s~~nd toxicity (ADMET). A comparative assessment was carried out with pharmacological and docking scores of the known effective

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wWithanolide A and wWithaferin A to make predictions of withanolides obtained from *D. innoxia*. As the chemo-informatics screening ~~remakedly~~notably, proved the effectiveness of dDinoxin B, its antibacterial properties ~~a~~were evaluated using pathogenic strains of *S. aureus* and *E. coli*.

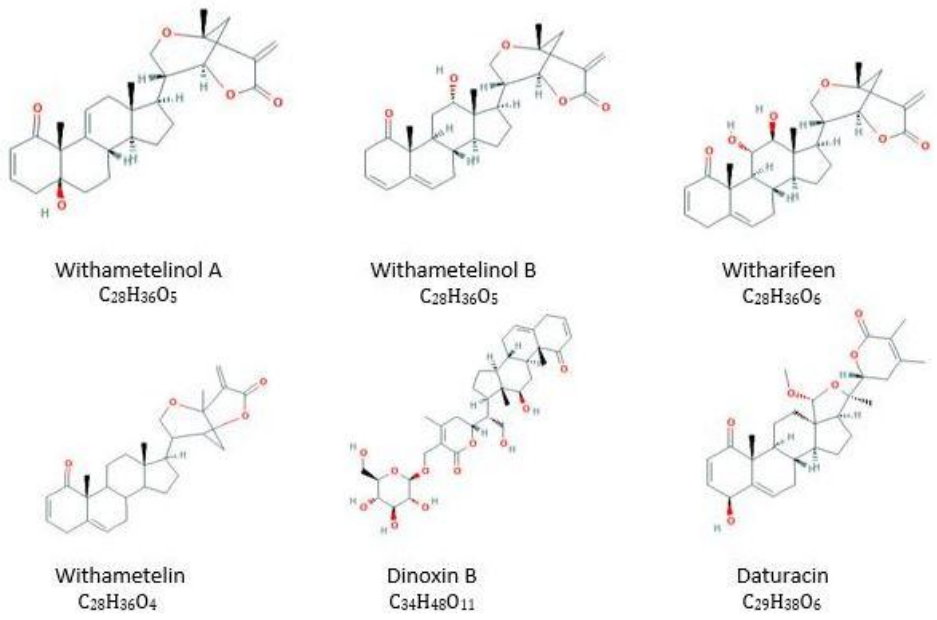

Fig. 2.: Molecular structure of withanolides of *D. innoxia* retrieved from PubCchem.

2. Methodology

2.1 Physicochemical Pproperties Pprediction

The SwissADME (https://swissadme.ch) tool wais used to examine the molecular properties and drug likeliness of withanolides based on Lipinski's rRule of five. ~~Lipinski's~~ This rule ~~of five~~ is used by pharmaceuticals in drug development to predict the oral bioavailability of potential lead or drug molecules [16]. These~~drug molecules[16]. These~~ parameters include total polar surface area (TPSA), partition coefficient (water/oil;) — cLogP), molecular weight, number of hydrogen acceptors, and number of hydrogen donors.

2.2. Pharmacokinetic Aanalysis

While ADME ~~tries~~ seeks to maximize the pharmacological performance of a small molecule, toxicology aims to eensure that it causes no harm in any kind of side effect [17]. In the present study, tThe obtained scores~~side effect[17]. Obtained scores~~ wereare comparatively analyzed with the scores of prevailing broad-spectrum antibiotics aAmpicillin, gGentamicin, and cCephalosporin. This program predicts based on functional group similarity of the investigated compound with the extensively *in- vitro* and *in- vivo* studied compounds present in its database.

2.3 Docking SStudies

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A docking study undertaken with standard precision mode using the Glide docking module of Maestro 12.5 Schrodinger [18] software was carried out to evaluate the affinity of withanolides towards *S. aureus* and *E. coli*. As shown in Table 1, different proteins responsible for resistance mechanisms were retrieved from the protein data bank, and ligands (withanolides) were retrieved from PubChem (Table 2).

Table 1: Details of selected proteins retrieved from the Protein data bank.

Proteins	(PDB ID)	
	S. aureus	*E. coli*
Penicillin Binding Protein	3 HUM	4BJP
DNA Gyrase	2XCT	1AB4
Efflux Pump Proteins	4 LLL	5ENO
Quorum Sensing Regulators	4G4K	2AVX

~~Table.1: Details of selected Proteins retrieved from the Protein data bank.~~

Table 2: Selected withanolides with Pub-Chem ID.

Ligands	Pub-Chem ID
Withametelinol A	15550331
Withametelinol B	101160729
Withrifeen	12135064
Daturacin	16010830
Withametelin	364746
Dinoxin B	51041991
Withanolide A	11294368
Withaferin A	265237

~~Table.2: Selected withanolides with its Pub Chem ID.~~

2.4 Extraction and Identification of Dinoxin B from *D. innoxia*

Following the protocol of Tandon et al. [10], ethanolic leaf extracts of *Datura innoxia* were fractionated using a single solvent system through column chromatography [19]. To fill up the column, silica gel (60–120 mesh) was used and added with the leaf extract, and the collection of the fraction was done by pouring solvent at a flow rate of 1ml/minute until silica gel became visible as colorless. For the identification of Dinoxin B, Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS) of fraction 4 [10] was done from the Central Drug Research Institute of India, Lucknow.

2.5 Agar Diffusion Assay

Following the Kirby–Bauer diffusion technique [20], we conducted an agar well plate method to assess the antibacterial property against clinical strains of *S. aureus* (SU-6151) and *E. coli* (EU-6081) isolated from urine samples of patients, including methicillin-resistant *S. aureus* (SU-6089) and multi-drug resistant *E. coli* (EU-6089). All isolates were obtained from the Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India, and the inhibition zones (ZOI) were then measured.

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Gentamicin (85 mg) and DMSO (10%) were used as positive and negative controls. Assays were done in triplicate, and the results were expressed as mean standard deviation.

2.6 Macrobroth Dilution for Determining MIC and MBC

Using the two-fold serial dilution procedure, different concentrations of fraction four were obtained and combined with 100 µl of the test organism to achieve a final inoculum concentration of 5 x 10^5. The minimum inhibitory concentration (MIC) value was defined as the highest dilution that inhibited bacterial growth. The growth control was the bacterial inoculum without a tested percentage, while the sterility control was the bacterial inoculum itself.

Minimum bactericidal concentration (MBC) was determined by subculturing each MIC tube that had no apparent growth. At 37°C, the plates were incubated for 24 hours. MBC refers to the lowest concentrations of the extract that did not result in colony formation on the solid medium.

3. Results and Discussion

Herbal drug formulation depends on its high biological potentiality and low toxicity. For oral absorption in terms of permeability, Lipinski and collaborators [16] have proposed that orally active compounds should fit at least three of the observed four parameters: molecular weight < 500 g mol^{-1}, log*P* < 5; number of hydrogen bond acceptors <10; number of hydrogen bond donors <10; and the well-known Lipinski's rule of 5 (Ro5). That is, Ro5 indicates a physicochemical space in which molecules outside its domain have a low probability of becoming orally active [21]. The Swiss ADME was employed to study Lipinski's rules for withanolides, and it was noted that all selected compounds were orally active compounds, as they satisfy more than three Ro5 parameters (Table 3).

Table 3. Results showing physiochemical properties to predict the drug-likeness of selected withanolides based on Lipinski's Ro5. TPSA: Total Polar Surface Area; nV: No. of Ro5 violations; ROTB: no. of rotatable bonds; MV: Molecular weight.

Compound	TPSA (A^0)	H bond Acceptors	H-bond Donors	cLogP	nV	nROTB	MV
Dinoxin B	183.2	11	6	1.71	3	7	632.75
Withanolide A	96.36	6	2	4.15	0	2	470.61
Withametelinol-A	72.84	5	1	4.51	0	1	452.6
Withametelinol-B	72.84	5	1	4.54	0	1	452.6
Witharifeen	93.07	6	2	3.62	0	1	468.59
Withametelin	52.61	4	0	4.95	0	1	436.59
Withaferin A	116.5	7	3	3.18	0	2	486.61

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The pharmacokinetic properties such as absorption, distribution, metabolism, excretion, and toxicity of withanolides were predicted by using Pre-ADMET and are shown in Table 4. The Caco-2 ~~cell model~~ and MDCK (Madin–Darby canine kidney) cell models have been accepted as reliable *in vitro* models for the assessment of oral drug absorption [22]. All observed withanolide showed middle permeability as per the Caco-2 and MDCK cell models, with a value between 4–70 and > 0, respectively.

~~HIA (~~Human ~~i~~Intestinal ~~a~~Absorption (HIA) and skin permeability models can also forecast and discover prospective medications for oral and transdermal delivery *in silico*. The sum of bioavailability and absorption in humans is calculated using the ratio of excretion or cumulative excretion in urine, bile, and feces [23]. Selected withanolides are recorded as well absorbed compounds ~~wit~~when they have~~h more than 90%~~ an ~~a~~of ~~a~~absorption rate of over 90% (HIA 70–100%). More HIA values indicate that the withanolides could be better absorbed from the intestinal tract upon oral administration. At the same time, the negative skin permeability of compounds predicts their poor transdermal properties. As shown in Table 2, the absorption rate of ~~W~~withametelin was~~is~~ ~~mor~~higher in all cases.

Table 4. Results of absorption properties ~~y of~~ of withanolides obtained from PreADMET.

	Absorption			
Compound	Caco-2 (4–70%; Middle permeability)	HIA (70–100%; well-absorbed compounds)	Skin Permeability (> 0 Poor Skin permeability)	MDCK (> 0 shows permeability)
Withametelinol A	30.683	96.388853	-1.85921	0.059029
Withametelinol B	28.314	96.390242	-2.94982	0.103376
Witharifeen	21.682	94.789426	-3.79095	0.178691
Withametelin	46.749	97.401304	-2.21215	0.052234
Dinoxin B	20.5044	92.577878	-4.69651	0.072432
Withanolide A	22.0036	94.739698	-2.63567	0.047720
Withaferin A	20.9127	90.403397	-3.75156	0.148721

~~Table.4:Result of absorption property of withanolides obtained from PreADMET.~~

In distribution, predicting ~~BBB (b~~Blood–~~B~~brain ~~b~~Barriers (BBB) ~~means~~ is done to indicate whether compounds pass across ~~the~~ blood-brain barriers. CNS-active compounds must pass across ~~it~~ the BBB, while~~and~~ CNS-inactive compounds must not pass across this barrier~~it~~ [24]. All~~across it[24]. All~~ ~~W~~withanolides with less BBB permeability are considered ~~as~~ CNS inactive compounds, which promote its drug-likeness with fewer CNS side effects.

It is generally assumed that only a~~the~~ free drug can cross membranes and bind to the intended molecular target [25], and thus~~molecular target[25] and~~ it is~~, therefore,~~ important to estimate the fraction of drugs bound to ~~p~~Plasma ~~p~~Protein ~~b~~Binding (PPB). PPB values greater than 90% indicate that they are strongly bound to plasma proteins. ~~and~~ ~~A~~all withanolides in the present study showed remarkable PPB efficiency; notably, withametelin showed a ~~hundred percentage~~100% binding affinity (Table 5).

A soluble compound promotes drug formulation, as solubility is an important criterion that may alter the effectivity of a drug compound. Withametelin, ~~W~~withametelinol A, and ~~D~~dinoxin B with high buffer

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solubility (73.47,79.83,66.44mg/l) and pure water solubility (40.5,33.51,50.09 mg/l) significantly ~~remarkably~~ proved their drug ~~-~~likeness.

Table ~~.~~5. ~~:~~Results showing the distribution property of withanolides in pharmacokinetics.

Compound	Buffer Solubility (mg/L)	Pure water Solubility (mg/L)	BBB	Plasma Protein Binding
Withametelinol A	79.8352	33.51143	0.188225	96.545118
Withametelinol B	47.511	28.41036	0.0927233	89.49667
Witharifeen	55.047	18.49928	0.143512	86.889916
Withametelin	73.4706	40.5095	0.310565	100
Dinoxin B	66.4456	50.0991	0.0483601	91.017295
Withanolide A	33.7106	35.22969	0.336643	91.590333
Withaferin A	3.31074	33.5959	0.159809	82.408739

~~Table.5:Result showing the distribution property of withanolides in pharmacokinetics.~~

~~For a better~~To improve the selection of drug compounds, knowledge about the interaction of molecules with cytochrome P450 (CYP) is essential [26]. ~~Itwith cytochromes P450 (CYP)is very essential[26]. It~~ has been proposed that CYP can synergistically metabolize tiny compounds to promote tissue and organism protection. Five main isoforms are thought to be the substrate of 50 ~~to~~ 90% ~~percent~~ of therapeutic compounds (CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4). The ~~i~~Inhibition of these isoenzymes is undoubtedly one of the most common causes of pharmacokinetics-related medication ~~-~~drug interactions, which can result in toxic or other undesirable side effects due to decreased clearance and buildup of a~~the~~ drug or its metabolites (de Montellano, 2015). Swiss ~~-~~ADME enables the estimation of the withanolides to be the substrate of P-gp or the inhibitor of the most important CYP isoenzymes. *In ~~-~~silico* data estimated that the selected withanolides ~~cannot~~ could not metabolize (non-substrate) by CYP 450 2D6 and were~~are~~ the substrate of CYP 450 3A4 non-inhibitors for CYP 450 2C19 and~~,~~ CYP 450 2D6, and the inhibitor of CYP 450 3A4 (Table 6). The noninhibition of cytochrome P450 was shown to~~ill~~ help in the metabolism of these compounds.

Table ~~.~~6. ~~:~~Result of metabolism prediction of withanolides using SwissADME~~dme~~.

Compound	CYP2C19 ~~i~~Inhibition	CYP2C9 ~~i~~Inhibition	CYP2D6 ~~i~~Inhibition	CYP2D6 ~~S~~Substrate	CYP3A4 ~~I~~inhibition	CYP3A4 ~~S~~Substrate
Withametelinol A	Non	Inhibitor	Non	Non	Inhibitor	Substrate
Withametelinol B	Non	Inhibitor	Non	Non	Inhibitor	Substrate
Witharifeen	Non	Inhibitor	Non	Non	Inhibitor	Substrate

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Withametelin	Non	Inhibitor	Non	Non	Inhibitor	Substrate
Dinoxin B	Non	Inhibitor	Non	Non	Inhibitor	Substrate
Withanolide A	Non	Inhibitor	Non	Non	Inhibitor	Substrate
Withaferin A	Non	Inhibitor	Non	Non	Inhibitor	Substrate

~~Table.6:Result of metabolism prediction of withanolides using SwissAdme.~~

The Ames test is a simple method for determining a compound's mutagenicity that was proposed by Dr. Ames. It employs several Salmonella typhimurium strains that have mutations in genes implicated in histidine synthesis, requiring histidine for growth. The mutagenic capacity to cause a reversion to growth on histidine-free media is being investigated. As shown in Table~~.~~ 6, ~~I~~the *in-vitro* Ames test results in the TA100 ~~strain~~, TA10010, TA153510 ~~strain~~, and TA1535 strains ~~are~~did not show~~ed~~ metabolic activation by rat liver homogenate. The~~is~~ Ames~~MES~~ toxicity results proved~~s~~ that all withanolides in the present study were~~are~~ non-mutagenic, with negative results (Table~~.~~ 7).

Table 7: Result of toxicity prediction of withanolides based on Ames t~~T~~est using Swiss-ADME.

Compounds	Ames Test	Oral Rat Acute Toxicity	Oral Rat Chronic Toxicity	TA10010 RLI	TA100 NA	TA153510 RLI	TA1535 NA
Withametelion A	non-mutagen	2.094	0.344	negative	negative	negative	negative
Withametelion B	non-mutagen	2.234	1.715	negative	negative	negative	negative
Witharifeen	non-mutagen	2.319	1.685	negative	negative	negative	negative
Withametelin	non-mutagen	2.045	1.171	negative	negative	negative	negative
Dinoxin B	non-mutagen	3.555	2.86	negative	negative	negative	negative
Withanolide A	non-mutagen	2.913	1.739	negative	negative	negative	negative
Withaferin A	non-mutagen	3.503	2.154	negative	negative	negative	negative

~~Table.7: Result of toxicity prediction of withanolides based on Ames Test using Swiss ADME.~~

To identify the target of these withanolides, docking interaction was observed with resistance promoting ~~M~~membrane proteins and ~~C~~chromosomal proteins (DNA g~~G~~yrase) of *S. aureus and E. coli*, as ~~given~~ shown in ~~T~~Table 1. Molecular docking is an effective method to predict the binding target of protein–ligand complexes and to determine the potential mechanisms of action. ~~O~~The ~~obtained~~ results justif~~ied~~y the antibacterial assays obtained, in which the binding affinity of withametelin [14] and ~~of withametelin[14] and~~ d~~D~~inoxin B [10] were shown to be ~~are~~ bacteriostatic. Docking results showed that d~~D~~inoxin B possessed~~s~~ a significant binding affinity to membrane proteins compared to~~than~~ DNA g~~G~~yrase (1 AB4 and 2XCT) with a docking score of -6.504 with e~~E~~fflux p~~P~~ump p~~P~~rotein (EPP) of *E. coli* and a docking score of -5.92 with p~~P~~enicillin-b~~B~~inding p~~P~~rotein (PBP) of *S. aureus*. (Fig. 2). Notably, this binding score

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was higher than wWithanolide A and wWithaferin A. As a common target, all these withanolides in the present study showeds better affinity towards PBPs (3HUM and 4BJP) and EPP-s of *E. coli*, which in turn indicateds membrane protein interaction as the reason for the antibacterial mode of action (Fig. 3).

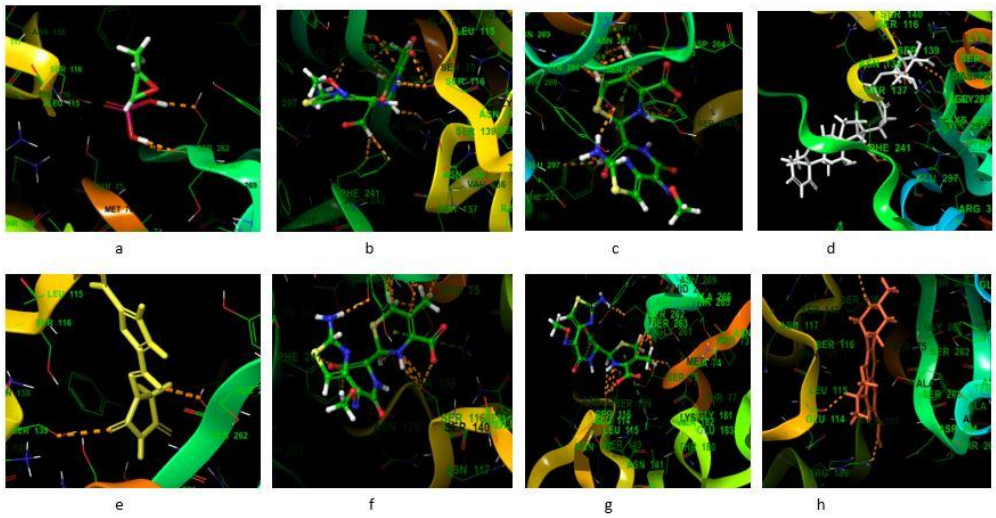

Fig. 3. The results showing the best protein–ligand interaction in the docking study. (a) Withametelin. (c) Wwithanolide A. and (d) Ddinoxin B with 3HUM(PBP) of *S. aureus*. (e) Wwithanolide A. (f) Withaferin A. (g) Wwithametelinol E.b (h) Ddinoxin Bb with 5ENO (EPP) of *E. coli*. (b) Wwithametelin with 4BJ (PBP) P of *E. coli*. PBP: pPenicillin binding protein; EPP: eEfflux pump protein.

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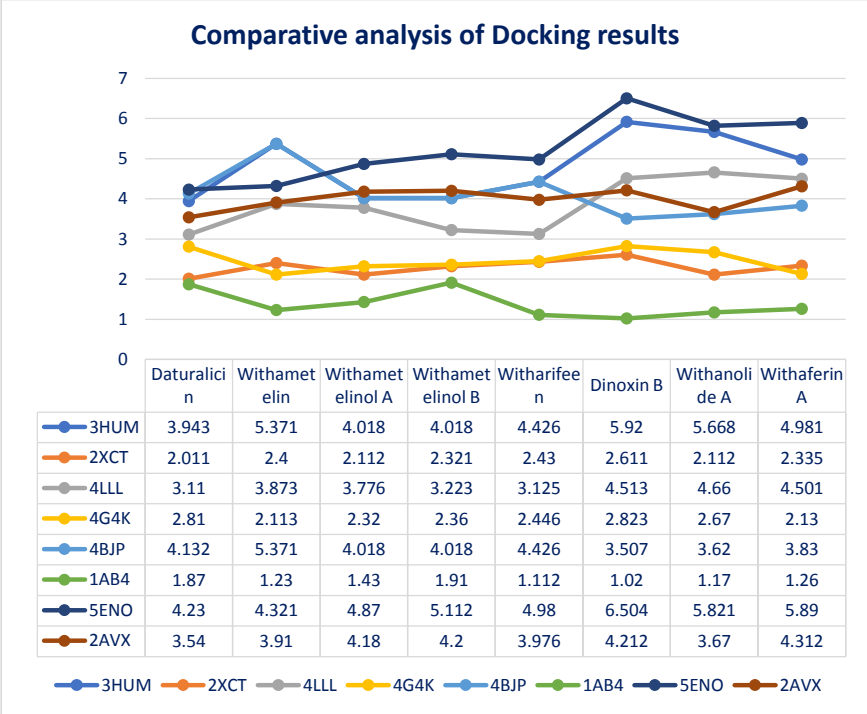

	Daturalicin	Withametelin	Withametelinol A	Withametelinol B	Witharifeen	Dinoxin B	Withanolide A	Withaferin A
3HUM	3.943	5.371	4.018	4.018	4.426	5.92	5.668	4.981
2XCT	2.011	2.4	2.112	2.321	2.43	2.611	2.112	2.335
4LLL	3.11	3.873	3.776	3.223	3.125	4.513	4.66	4.501
4G4K	2.81	2.113	2.32	2.36	2.446	2.823	2.67	2.13
4BJP	4.132	5.371	4.018	4.018	4.426	3.507	3.62	3.83
1AB4	1.87	1.23	1.43	1.91	1.112	1.02	1.17	1.26
5ENO	4.23	4.321	4.87	5.112	4.98	6.504	5.821	5.89
2AVX	3.54	3.91	4.18	4.2	3.976	4.212	3.67	4.312

Fig. 4. Comparative analysis of docking results of withanolides of *D. innoxia*.

As we reported in a previous study [10], ethanolic~~in the previous study[10], ethanolic~~ leaf fraction four of *D. innoxia* obtained through column chromatography ~~i~~was analyzed through LC-ESI-MS. This mass spectrum (Fig. 5) also depicted~~s~~ the presence of d~~D~~inoxin B w~~W~~ithanolide and its aglycone. Phytoconstituents eluted in the spectrum of fraction four depicted as~~-~~ M-glucose-water+H^+ (*m/z*471) and Dinoxin B Withanolide (*m/z* 633) due to the cleavage of a glycosidic bond.

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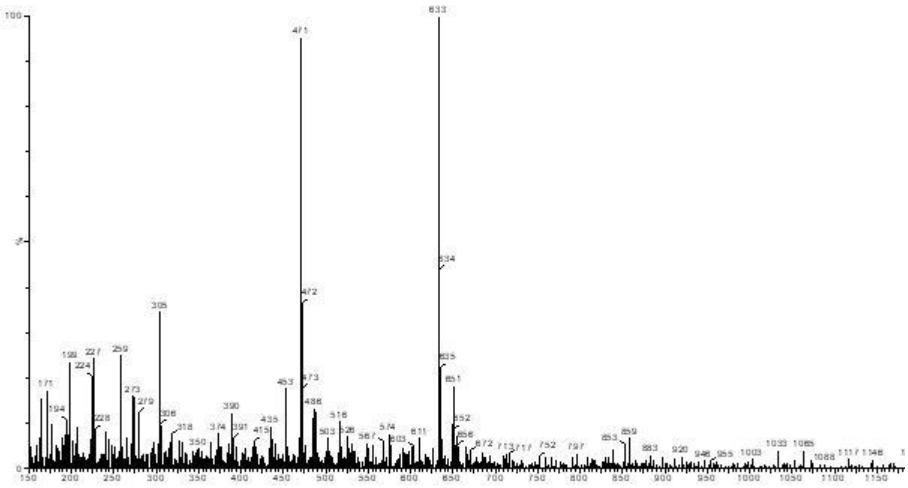

Fig. 5. LC-ESI-MS spectrum of most active fraction four showing the presence of dinoxin B withanolide and its aglycone. The phytoconstituents eluted as M-glucose-water+H$^+$ (*m/z*471) and dinoxin B withanolide (*m/z* 633).

The inhibitory potential of dinoxin B was observed through agar well diffusion assay using different concentrations of fraction four in µg/ml (100,000, 50,000, 25,000, and 12,500) and compared to the control (DMSO) and gentamicin as reference antibiotics (Fig. 6). As per the Kirby–Bauer test [27], *S. aureus* susceptibility~~Kirby-Bauer test[27], *S.aureus* susceptibility~~ based on ~~Zone of Inhibition~~the ZOI was evaluated (< 12 mm [~~r~~resistant]; <13–14 mm [~~i~~intermediate]; and > 15 mm [~~s~~susceptible]). As shown in Figure 6, clinical strains ~~(~~ (U-6151 and U-6081) ~~-~~ isolated from urine samples, including MRSA (U-6089), and MDR (U-6089), ~~were~~ showed significant activity ($p < 0.05$), which was comparable to the reference antibiotic, at a higher concentration of dinoxin B (100,000 µg/ml, 50,000 µg/ml). Whereas the MDR strain at 25,000 µg/ml and 12,500 µg/ml, as well as~~d~~ MRSA at 12,500 µg/ml showed low levels of susceptibility. Susceptibility decreased with a decrease in concentration, which showed~~s~~ the impact of dinoxin B in ~~its~~ higher concentrations. ~~The zone of inhibition~~ZOI varied (Table 8) in the range of (mm) 0–15 (1250 µg/ml), 0–18 (25,000 µg/ml), 9.1–20.3 (50,000 µg/ml), and14.6–23.3 (100,000 µg/ml), in which MDR (U-6090) showed higher resistance. Dinoxin B showed higher susceptibility to the methicillin-resistant strain (U-6089) than ~~that of~~did gentamicin, with a 22.5 mm ~~zone of inhibition~~ZOI.

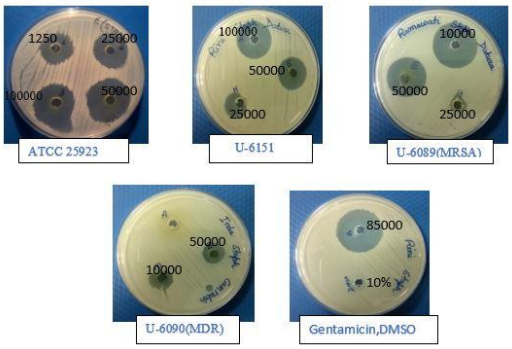

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Fig. 6. ZOI (mm). *S. aureus* strains showing susceptibility to different concentrations of dinoxin B with DMSO (control) and gentamicin (reference antibiotic).

Table 8. ZOI (mm) for dinoxin B. Data is in triplicate and represented as mean ± SD. < 12mm (resistant); < 13–14mm (intermediate), and > 15mm (susceptible); shown as (R), (I), and (S).

S. aureus	Different conc. Of Fraction 4 (µg/ml)				Gentamicin	DMSO
	12,500	25,000	50,000	100,000	(85,000) (µg/ml)	(10%)
ATCC25923	15±0.5 (S)	18±1(S)	20.3±1.0(S)	23.3±0.7(S)	26.1±0.2(S)	0
U-6151	15.8±0.7(S)	16.2±0.5(S)	21.6±0.5(S)	24 ±0.5 (S)	25.8±0.5(S)	0
U-6081	18.6±0.5(S)	15.4±0.5(S)	21.3±0.5(S)	22.5±0.5(S)	22.8±0.28 (S)	0
U-6089 (MRSA)	0(R)	3.4±0.5(R)	19.3±0.5(S)	22.5±0.5(S)	22.8±0.28(S)	0
U-6090 (MDR)	0(R)	0(R)	9.1±0.28(R)	14.6±.28(S)	14.2±0.28(S)	0

Antibacterial effectiveness was evaluated through MIC assay, in which the maximum dilution of dinoxin B that slowed down staphylococcal growth was noted. As shown in Table 9, dinoxin B showed the same levels of MIC (12.5 ±0.00) against all strains of *S. aureus* except the MDR strain (50±0.00). With a lower MIC (12.5±0.00) against UTIs, dinoxin B can be considered a potent phytocompound. The growth of bacteria was not inhibited in the negative controls. The minimum bactericidal concentration (MBC) of dinoxin B was found to be 25±0.00 mg/ml by the absence of bacterial colonies on fresh Muller–Hinton agar plates.

Table 9. Results of MIC, MBC, and MIC/MBC. Data is in triplicate and represented as mean ± SD. nd: not determined.

S. aureus **Strains**	**MIC (mg/ml)**	**MBC (mg/ml)**	**MBC/MIC Ratio**	**Bactericidal(+) Bacteriostatic(-)**
ATCC 25923	12.5±0.00	25.0±0.00	2	+
U-6151	12.5±0.00	25.0±0.00	2	+
U-6081	12.5±0.00	25.0±0.00	2	+
U-6090 (MDR)	50±0.00	>100	nd	nd
U-6089 (MRSA)	12.5±0.00	25.0±0.00	2	+
Gentamicin	12.5±0.00	25.0±0.00	2	+
DMSO	0	0	0	0

The MBC/MIC ratio ≤ 2 indicated bactericidal effects, and the MBC/MIC ratio ≥ 4 indicated bacteriostatic effects. Accordingly, dinoxin B was found to have bactericidal effects against all tested isolates of *S. aureus* except the MDR strain (U-6090), as shown in Table 2.

4. Conclusion

Withanolides are a class of active compounds widely found in Solanaceae plants. In traditional applications, they have a long history and a wide range of functions. With the progress of drug

structure and pharmacological research, ~~more and more~~ reports increasingly point to their~~its~~ excellent pharmacological effect~~s~~. Based on the pharmacological action of inhibiting bacterial resistance, withanolides have become a research hotspot in natural medicine. The use of *i~~I~~n n-silico* results ~~help us to~~allowed us to conclude that d~~D~~inoxin B and w~~W~~ithametelin can be considered ~~as~~ drug candidates due to their relevant ~~D~~drug -likeness and adequate pharmacokinetic~~s~~ features. Dinoxin B, with its significant antibacterial properties, emphasizes the therapeutic~~al~~ potential~~ity~~ of *D. innoxia*.

COMPETING INTERESTS DISCLAIMER:

A~~a~~The authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use ~~pro~~ducts in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. In addition~~Also~~, the research was not funded by the producing company, rather it was funded by the personal efforts of the authors.

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