Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 74 (2): 50-66 Jul 2022
DOI: 10.5455/ajvs.50029

Phytochemical Analyses, Antimicrobial Activities and Possible Antimicrobial Mechanism of Adansonia Digitata: An Animal Model

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Key words:
Antimicrobial, vegetables, resistance, .microbes, mechanism

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Article History
Received: 11 March 2022
Accepted: 01 July 2022

ABSTRACT

The advent of antibiotics recorded a huge amount of success against disease-causing microbes. However, development of resistance to reference antibiotics leading to increased mortality have caused critical concern in the management of infectious diseases. Continuous search for alternative therapies has shown good candidates in medicinal plants, although without thorough examination of possible mechanism of action. This study assessed the phytochemical constituents, antimicrobial activities, and possible antimicrobial mechanism of *Adansonia digitata*, against *Shigella flexneri*, *Escerichia Coli* and *Salmonella enteritis*. Phytochemical analyses showed that *Adansonia digitata* is rich phytosterols, flavonoids, phenolics and carotenoids. Assessments of antimicrobial activities of *A. digitata* revealed its effectiveness against *S. flexneri, E. coli* and *S. enteritis*. The mechanism of antimicrobial activities of *A. digitata* may involve inhibition of Gram+ and Gram- transpeptidase and dihydrofolate reductase. The antimicrobial activities may be ascribed to the presence of phytochemicals in *A. digitata*.

1. INTRODUCTION

In developing countries, many individuals depend mainly on alternative therapies for their healthcare. This may be due to resistance of the microorganisms to the reference drugs, high cost of treatment due to expensiveness of drugs, unavailability of the reference drugs and observed side effects of the synthetic drugs. Also, the increase in resistance of the microbes to most recommended antibiotics led to increased ineffectiveness of the drugs, high cost of treatments, and increase in mortality. (Fair and Tor, 2015; Opperman and Nguyen, 2015). The continous observation and reports of the limitations had resulted into search for alternative sources of therapies which may be less expensive, have little or no side effects and that will be readily available (Adekunle et. al., 2016). Functional foods have been defined to be those foods that contain, apart from basic nutrients such as protein, carbohydrates, vitamins, lipids, and minerials, certain bioactive compounds that are of health benefits to man. One of the categories of the functional foods is plant source and examples are vegetable. What confers the health benefits on the functional foods is the presence of phytochemicals such as tannins, terpenoids, alkaloids, flavonoids etc which have been found in vitro to have antimicrobial properties (Talib and Mahasneh, 2010). *Adansonia digitata is* a plant commonly consumed as vegetable in West African

region. Information on antimicrobial activities of this is rare.

Although, there have been several reports of possible antimicrobial activities of medicinal plants, however, few have proposed possible mechanism of action. The understanding of the mechanism of antimicrobial action of medicinal plants extract is the first step in the optimal utilization of these extracts as natural antimicrobial agents. This study is designed to assess the phytochemical constituents, investigate possible antibacterial activities and possible antimicrobial mechanism of *Adansonia digitata*.

2. MATERIALS AND METHODS

In this study, the antimicrobial efficacy of Adansonia digitata was tested against E. coli, shigella flaxneri, and salmonella enteritis infections. Fifteen rabbits were used and categorized into 3 equal groups as follows: group 1consisted of 5 rabbits infected with E. coli and later treated with aqueous extract of A. digitata. Group 2consisted of 5 rabbits infected with salmonella enteritis and later treated with aqueous extract of A. digitata. Group 3consisted of 5 rabbits infected with Shigella flaxneri and later treated with aqueous extract of A. digitata.

2.1 Animal study

2.1.1 Bacteria culture and inoculation of animals

The organisms were cultured on different media. A pure colony was picked for each organism and mixed with 9ml of sterile distilled water. This gave a concentration of 10^{-1}. Sterile pipette was used to remove 1ml of the dilution and 9ml of sterile water was added to give 10^{-2}. From 10^{-2} dilutions, 2ml was orally given to the rabbits as the innoculum size. Group 1 was infected with E. coli, group 2 was infected with Salmonela enteritis and group 3 was infected with Shigella flaxneri. Also, their feeds were mixed with the organisms to ensure infection. After 24 hours post challenge, specimen such as stool was collcted from the animals and cultured to ascertain that they have been infected with the organisms. After infection has been confirmed, parameters such as weight, temperature, packed cell volume and presence of bacteria in stool samples were examined again. The essence was to determine any alteration during infection. Blood samples were collected for 24 hours post challenge analyses of biochemical parameters (infected state)

2.1.2 Administration of aqueous extracts of plants

Two milliliters of aqueos extract of A. digitata was administered to infected rabbits in groups 1, 2 and 3 respectively. The administration was twice daily i.e. morning and evening for three(3) consecutive days. By 72 hours, effectiveness of the extract was determined by culturing stool samples for the presence of organisms. The organisms were observed to be absent from stool cultures. After confirmation of absence of microorganisms, post treatment examination of the stools, packed cell volume, weight, temperature and biochemical parameters were again carried out.

2.1.3 Determination of packed cell volume

Blood at various stages of study i.e. at baseline, during infection and post-infection were taken from animals by bleeding the rabbits. The packed cell volume (PCV) was measured using whole blood directly from animals, and was carried out using the Heamatocrit method described by Schalm et. al., 1975).

2.1.4 Determination of biochemical parameters

The serum total proteins and albumin were determined spectrophotometrically. Globulin levels were determined by calculation.

2.2.0 Chemicals and reagents for phytochemical determination

All chemicals and reagents are of analytical grade. Bis(trimethylsilyl)trifluoroacetamide, hexane, benzene, petroleum ether, sodium hydroxide, hydrochloric acid, ethanol, potassium hydroxide, acetone, ammonium acetate were purchased from Sigma chemical Company. All chemicals were used without further purification.

2.2.1 Qualitative and quantitative phytochemical analyses

Qualitative phytochemical analyses were carried out on the powdered form of dried Adansonia digitata to identify the presence of secondary metabolites. The bioactive compounds tested for are; Phenolics, Phytosterols, Flavonoids and Carotenoids. Phytosterol was extracted and analyzed using method of AOAC International. (Official Method 994.10), Phenolic and flavonoid contents were extracted and analyzed according to method described by Laghari et. al., 2011. The Carotenoids were extracted and analyzed by method described by Berhane et al., 2021.

Fig.1. *(Adansonia digitata)*

2.2.2 Determination and characterization of Phenolics and Flavonoids

Free phenolic and flavonoid contents were determined using High performance liquid chromatography (HPLC-DAD). This was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1. Briefly, reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm x 250 mm) packed with 5µm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 65 min, respectively, following the method described by Laghari et al. (2011) with slight modifications. The extracts of the 3 vegetables were analyzed, at a concentration of 5 mg/mL. The presence of phenolics compounds and the flavonoids were investigated. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards.

2.2.3 Determination and characterization of carotenoids

Analysis was carried out in an Agilent 1100 Model HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) fitted with a Hewlett-Packard Model 1050 solvent delivery system, an auto-sampler, and a UV-Vis detector (Santa Clara, CA, USA). It included a reversed-phase column YMC Carotenoid (250 × 4.6 mm I.D., S-5 µm, Japan), maintained at 35 ◦C. Chromatographic separation was performed using gradient elution with a tertiary mobile phase of MeOH-10 mM ammonium acetate, MTBE (100%), and water (100%) set in reservoirs A, B, and C, respectively. Each eluent for HPLC was filtered through a 0.45-µm membrane, and degassed ultrasonically for 5 min before use. Separations were performed by the following solvent gradient: 0–24 min 83.00% A, 15.00% B, and 2.00% C; 24–32 min 63.50% A, 35% B, and 1.50% C; 32–34 min 33.50% A, 66.00% B, and 0.50% C; and 35 min 83.00% A, 15.00% B, and 2.00% C. The total run time of the analysis was 45 min, including 10 min of system re-equilibration. The flow rate was 0.90 mL min−1, and 20.00 µL of the sample were injected into the HPLC system. Compounds present in the elution were monitored at 450 nm using a UV-vis detector. Peaks were identified by their retention time, and were compared to those of authentic standards. Data sets were collected, recorded, processed, and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Santa Clara, CA, USA).

2.2.4 Determination of phytosterols

The phytosterol extraction and analysis were carried out by following the modified method AOAC 994.10 and AOAC 970.51 Official Methods.

Five millgram (5.00g) of the powdered sample was weighed and transferred to Stoppard flask and treated with petroleum either until the powder was fully soaked. The flask shook every hour for the first six hours and then it was kept inside and shook after 24hours. This process was repeated for three days and then the extract was filtered. The extract was collected and evaporated to dryness by using nitrogen stream.

Zero point five gram (0.5g) of the extract from the sample was added to the screw-capped test tube. The sample was saponified at 95^{O}C for 30 minutes by using 3ml of 10% KOH in ethanol to which 0.20ml of benzene had been added to ensure miscibility 3ml of de-ionized water was added and 2ml of hexane was

used in extracting the non-saponifiable materials, eg., sterols. Three extractions, each with 2ml of hexane were carried out for 1 hour, 30min and 39mins respectively to achieve complete extraction of the sterols. The hexane was concentrated to 2ml in Agilent vial for gas chromatography analysis. Standards and samples were analyzed using the instrumental conditions shown in Table 1 below

2.3.0 In-silico study

This was done to investigate the binding and inhibitory capacities of each of the phytochemicals to critical proteins required for survival of the microorganisms. The study equally evaluated the transportation efficiency and toxicity properties of the phytochemicals in order to recommend the best candidate for antibiotic drug.

2.3.1 Method of Protein Preparation

After obtaining the X-ray crystallographic structures of the target proteins which include Dihydrofolate Reductase (DHFR) (PDB ID: 6vs6) and Transpeptidase (TP) gram + and gram - (PDB ID: 6i1e and 4gsu respectively) from the Protein Data Bank (PDB) (https://www.rcsb.org/) (Figure 1 and 2), we treated them accordingly using BIOVIA Discovery Studio Software version 19.1 in order to prevent unbidden molecular interactions during virtual screening. The active site was validated using both the reported publication and BIOVIA Discovery Studio reported residues for both target proteins (Bellini et al., 2019, Kim et al., 2013, Ribeiro et al., 2020). The amino acid residues reported by Biovia Discovery Studio include Leu 24, Gln28, Phe31, Arg32, Leu51, Val54, Leu57, Arg60 and Ile94 for DHFR, Ala288, Asn289, Ile293, Tyr330, Ser331, Gly383, Thr384, Leu385, Asp389, Leu391 for Transpeptidase (Gram+) and Tyr32, Val333, Ser349, Asn351, Tyr409, Lys484, Ser485, Thr487, Arg489, Tyr532, Phe533 for Transpeptidase (Gram-). Autodock tool-1.5.6 program (Morris et al., 2009) was used to determine the grids which include the dimension and binding centre of 6vs6 (63.989, 78.778, 26.433), 6ile (0.843, 43.025, 3.365) and 4gsu (-7.736, 36.293, -14.457).

2.3.2 Ligands Preparation

We used the fifty four (54) reported phytochemical compounds from the GC/MS analysis of four classes of phytochemicals which include Phenolics (Caffeic Acid: CID_689043, Chlorogenic Acid: CID_1794427, Cinnamic Acid: CID_444539, Ellagic Acid: CID_5281855, Ferrulic Acid: CID_445858, Gallic Acid: CID_370, O-Coumaric Acid: CID_637540, Protocatechuic Acid: CID_72, Salicylic Acid: CID_338, Sinapinic Acid: CID_637775, Syringic Acid: CID_10742, Vanillic Acid: CID_8468), Flavonoids (Epicatechin: CID_72276, Epigallocatechin: CID_72277, Apigenin: CID_5280433, Artemetin: CID_5320351, Baicalein: CID_5281605, Biochanin: CID_5280373, Butein

Table.1: GC conditions for the analysis of sterols

GC:	HP 6890 Powered with HP ChemStation Rev. A 09.01(1206) Software	
Injection Temperature:	Split Injection	
Split Ratio:		20:1
Carrier Gas:		Nitrogen
Inlet Temperature:	250^{O}C	
Column Type:		HP INNOWax
Column Dimensions:		30m x 0.25mm x 0.25um
Oven Program:	Initial Temperature @ 60^{O}C	
		First Ramping @ 10^{O}C/min for 20min, Maintained for 4min
		Second Ramping @ 15^{O}C/minfor 4min, Maintained for 10min
Detector:		FID
Detector Temperature:	320^{O}C	
Hydrogen Pressure:		22psi
Compressed Air:	35psi	

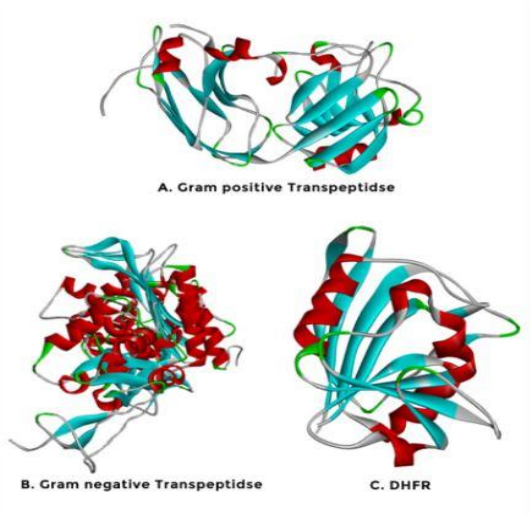

Fig 2: (A) The X-ray crystallographic structure of Gram+ transpeptidase **(B)** The X-ray crystallographic structure of Gram+ transpeptidase (C) The X-ray crystallographic structure of Dihydrofolate Reductase (DHFR)

: CID_5281222, Catechin: CID_73160, Daidzein: CID_5281708, Epicatechingallate: CID_107905, Gallocatechin: CID_65084, Genistein: CID_5280961, Isoquercitrin: CID_5280804, Isorhamnetin: CID5281654, Kaemferol: CID_5280863, Luteolin: CID_5280445, Myricetin: CID_5281672, Naringenin: CID_932, Naringin: CID_442428, Nobiletin: CID_72344, Quercetin: CID_5280343, Quecitrin: CID_5280459, Resveratrol: CID_445154, Robinetin: CID_5281692, Rutin: CID_5280805, Silymarin: CID_5213, Tangeretin: CID_68077, Vanillin: CID_1183), Carotenoids (Alpha-Carotene: CID_6419725, Antheraxanthin: CID_5281223, astaxanthin: CID_5281224, Beta-Carotene: CID_5280489, Lutein: CID_5281243, Lycopene: CID_446925, Neoxanthin: CID_5282217, Violaxanthin: CID_448438, Zeaxnathin: CID_5280899) and Phytostrols (Campesterol: CID_173183, Cholesterol: CID_5997, Ergosterol: CID_444679, Sitosterol: CID_222284, Stig-masterol: CID_5280794) in . They include The SMILES formats of the ligands were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), an open chemistry database, consisting of substance, compound, and bioassay (Kim et al., 2015). We converted them to 3-dimensional (3D) structures (.pdb format) for efficient virtual screening process using the online SMILES Translator at https://cactus.nci.nih.gov/translate webserver.

2.3.3 Molecular Docking Protocol

In this study, we used the PDB format of the target proteins (DHFR: 6vs6, TP gram+:6ile and TPgram-:4gsu) for the virtual screening while Auto Dock Vina Tools 1.5.6 was used for the protein's optimization, removal of crystallized water, addition of polar hydrogen and geisteiger charges. We executed the consensus molecular docking using three different softwares which are AutoDock Vina (Trott and Olson, 2010), Pyrex and IGMDock docking tools.

2.3.4 Pharmacokinetics (ADMET) and Drug-likeness Properties Evaluation

Molinspiration Online Tool (https://molinspiration.com/) was used to assess the drug-likeliness of the selected compounds while properties related to absorption, distribution, metabolism, excretion, and toxicity (ADMET) which depicts their pharmacokinetics and pharmacodynamics properties were evaluated using ADMET SAR2 webserver (https://lmmd.ecust.edu.cn/admetsar2/) (Yang et al., 2019).

2.4.0 Statistical analysis

Analysis of variance (ANOVA) was used for general comparison while student's t-test was used to determine the significance of differences in the selected physical, biochemical and haematological parameters before infection when compared with the status during infection (i.e. effect of infection). The differences were considered significant when the p-value was less than 0.05.

3.0 RESULTS

The results of these studies were presented using tables, figures and graphs. The results of the animal study showed the status of evaluated selected physical, hematological and biochemical parameters at pre-infection state (baseline), infection state and post-

infection state (post treatment). The changes in the status of all parameters assessed during infection when compared with baseline values reached level of statistical significance ($p \leq 0.05$). It was observed that during infection, the packed cell volume (PCV) and the weights of the rabbits were significantly reduced $p \leq 0.05$). However, after treatment of the different infections with the aqueous extracts, the observed alterations become normalized (tables 1,2 and 3). Results of the determination of the different phytochemicals, their characterization and quatifications reveals presence of phenolics (table 4 and figure 2), flavonoids (table 5 and figure 3), phytosteroids (table 6 and figure 4) and carotenoids (table 7 and figure 5).

Table 2: Showing the changes in the status of physical, hematological, and biochemical parameters assessed during *E. coli* infection and post- infection (after *Adansonia digitata* treatment) states.

Parameters	Before infection (Baseline)	During infection	Post-infection state (after treatment
Body weight	1.35±0.22	1.08±0.12	1.20±0.33
PCV	37.14±2.02	31.51±4.50	36.90±3.00
Temperature(^{0}C)	36.97±0.20	41.05±4.12	37.65±0.14
Total protein (mg/dl)	8.11±0.33	8.50±1.05	8.39±0.41
Albumin (mg/dl)	4.66±0.81	4.58±0.12	4.60±0.13
Globulin (mg/dl)	3.45±1.25	3.92±0.50	3.79±0.55

Significantly different from baseline ($p<0.05$).

Table 3: Showing the changes in the status of physical, hematological and biochemical parameters assessed during *salmonella enteritis* infection and post- infection (after *Adansonia digitata* treatment) states.

Parameters	Before infection (Baseline)	During infection	Post-infection state (after treatment
Body weight	1.38±0.11	1.23±0.09	1.33±0.13
PCV	36.40±0.15	39.10±0.11	37.20±0.31
Temperature(^{0}C)	34.40±4.16	37.00±2.15	36.1±2.05
Total protein (mg/dl)	6.10±1.00	7.21±0.21	6.50±0.10
Albumin (mg/dl)	3.17±0.52	3.20±0.33	3.33±0.01
Globulin (mg/dl)	2.83±0.44	4.01±0.11	3.17±0.12

Significantly different from baseline ($p<0.05$)

Table 4: Showing the changes in the status of physical, hematological and biochemical parameters assessed during *shigella flaxneri* infection and post- infection (after *Adansonia digitata* treatment) states.

Parameters	Before infection (Baseline)	During infection	Post-infection state (after treatment
Body weight	1.70±0.05	1.51±0.15	1.59±0.01
PCV	37.56±0.11	40.20±1.32	38.01±0.03
Temperature(^{0}C)	35.32±2.05	38.00±3.01	36.04±1.21
Total protein (mg/dl)	6.43±0.20	7.80±0.31	6.77±0.10
Albumin (mg/dl)	3.39±0.44	3.66±0.10	3.45±0.21
Globulin (mg/dl)	3.04±0.16	4.34±0.10	3.32±0.12

Significantly different from baseline ($p<0.05$)

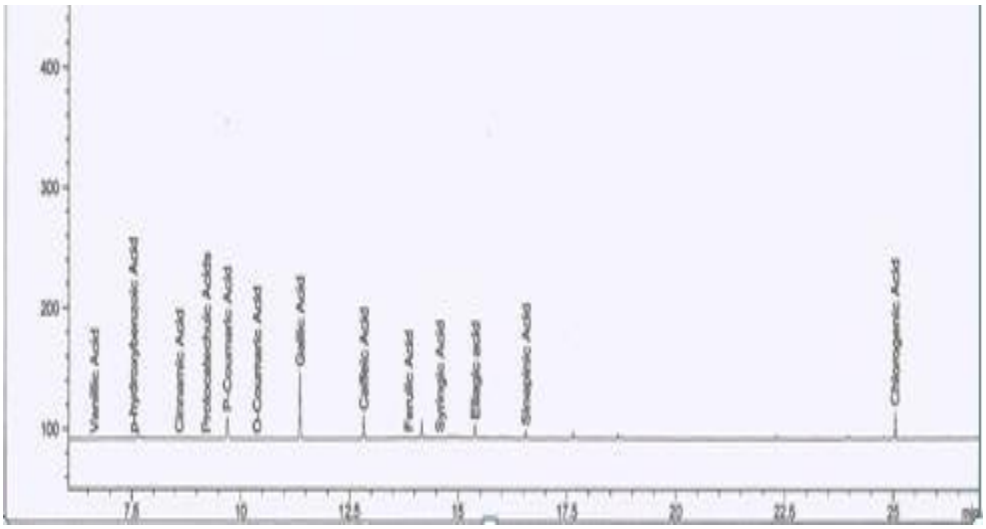

Fig 3: Chromatogram showing the phenolic acids present in *Adansonia digitala L* (baobab).

Table 5: Concentrations of phenolics present in *Adansonia digitala L.(*Baobab).

BIOACTIVE COMPOUNDS	AMOUNT (mg/100g)
Vanillic acid	1.001×10^{-1}
p-hydroxybenzoic acid	2.680
Cinnamic acid	3.410×10^{-7}
Protocatechuic acid	1.796×10^{-3}
P-coumaric acid	7.055×10^{-2}
O-coumaric acid	8.278×10^{-2}
Gallic acid	153.877
Caffeic acid	186.206
Ferulic acid	1.724×10^{-2}
Syringic acid	3.254×10^{-1}
Ellagic acid	73.330
Sinapinic acid	8.894×10^{-3}
Chlorogenic acid	219.889

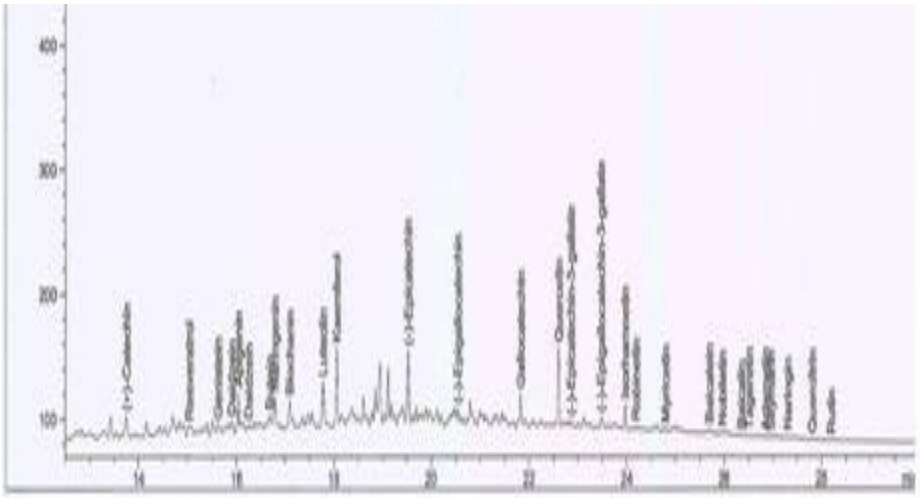

Fig. 4: Chromatogram showing the flavonoids present in *Adansonia digitala L* (baobab).

Table 6: Quantitative analyses of flavonoids present in Baobab.

BIOACTIVE COMPOUNDS	AMOUNT (mg/100g)
(+)-catechin	71.866
Resveratrol	1.272×10^{-5}
Genistein	1.347×10^{-5}
Daidzein	1.279×10^{-5}
Apigenin	4.753×10^{-3}
Daidzein	1.512×10^{-5}
Butein	2.449×10^{-5}
Naringenin	4.985×10^{-3}
biochanin	4.609×10^{-5}
Luteolin	255.910
Kaemferol	405.739
(-)-epicatechin	57.763
(-)-epigallocatechin	2.317×10^{-3}
Gallocatechin	1.489×10^{-4}
Quercetin	131.337
(-)-epicatechin-3-gallete	4.363×10^{-1}
(-)-epigallocatechin-3-gallate	5.062×10^{-4}
Isorhamnetin	6.138
Robinetin	2.201×10^{-5}
Myricetin	7.970×10^{-6}
Baicalein	1.674×10^{-5}
Nobiletin	1.663×10^{-5}
Baicalin	1.042×10^{-5}
Tageretin	4.816×10^{-6}

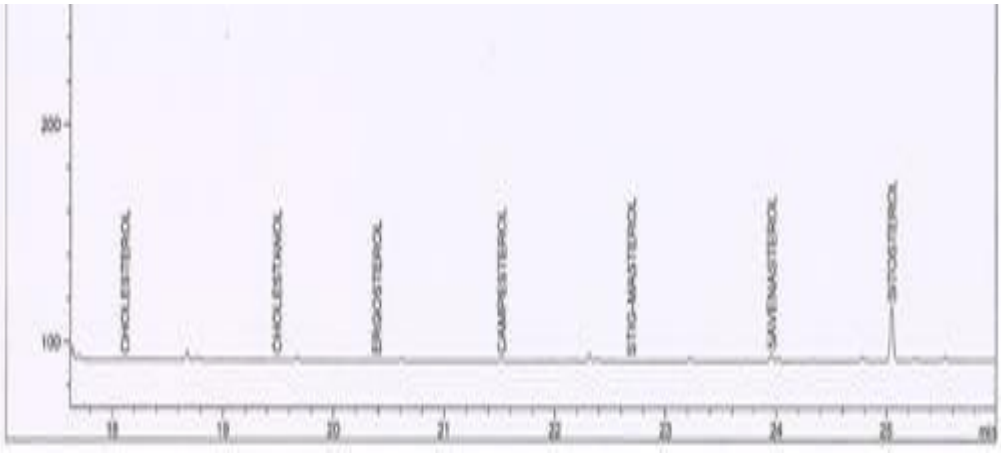

Fig 5: Chromatogram showing the phytosterols present in *Adansonia digitala L* (baobab).

Table 7: Concentrations of phytosterols present in *Adansonia digitala L* (baobab).

BIOACTIVE COMPOUNDS	AMOUNT (mg/100g)
Cholesterol	3.272×10^{-4}
Cholestanol	3.193×10^{-5}
Ergosterol	1.853×10^{-3}
Campestrol	4.176×10^{-1}
Stig-masterol	6.745×10^{-1}
Savenasterol	9.773×10^{-1}
Sitosterol	5.425

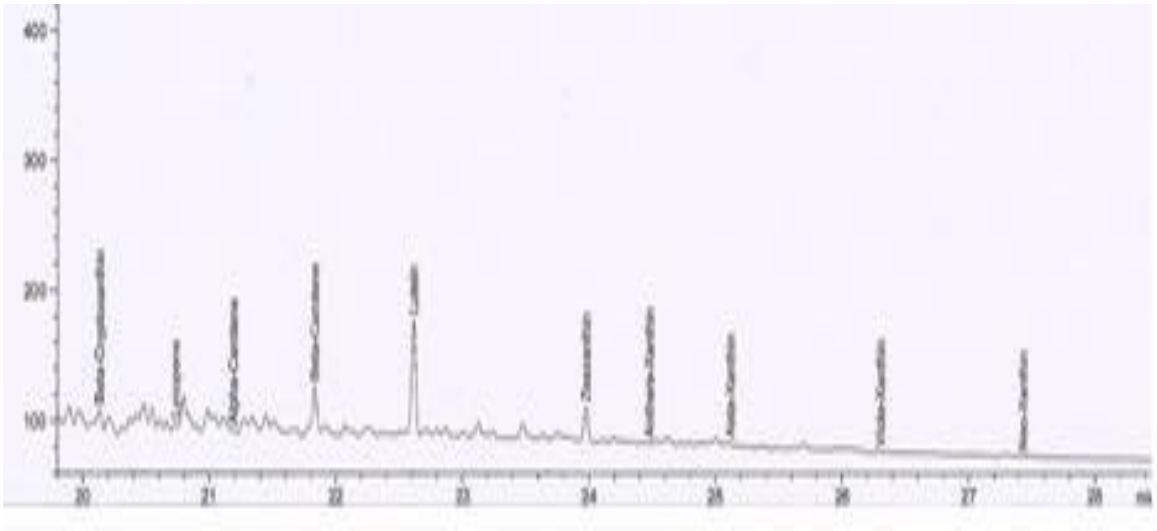

Fig. 6: Chromatogram showing the caroteniods present in *Adansonia digitala L* (baobab).

Table 8: Concentrations of the carotenoids present in the *Adansonia digitala L* (baobab).

BIOACTIVE COMPOUNDS	AMOUNT (mg/100g)
Beta-cryptoxanthin	1.649
Lycopene	6.304×10^{-6}
Alpha-carotene	6.304×10^{-6}
Beta-carotene	4.089
Lutein	5.936
Zeaxanthin	6.429
Anther-xanthin	2.079×10^{-1}
Asta-xanthin	4.369×10^{-1}
Viola-xanthin	1.633
Neo-xanthin	1.218

Table 9. Gram positive transpeptidase-carotenoids

S/N	Ligands	Consensus Binding Affinity	Conventional Hydrogen Bond	Other bonds
	Clavulanic Acid (Standard)	-6.63	Tyr318, His352	
	Penicillin (Standard)	-6.86	Tyr318	Met303, Val333
1.	Astaxanthin	-8.3	TYR 308, GLN 244	PHE 334, ILE 301, VAL 156
2.	Lycopene	-7.7	Nil	PHE 215, ILE 291, TRP 394, VAL 196, PRO 194, TRP 216
GRAM POSITIVE TRANSPEPTIDASE-FLAVONOIDS				
3.	Isorhamnetin	-7.8	THR 320, TRP 340	318, MET 303, CYS 354, ARG 319, HIS 352
4.	Epigallocatechin	-8.2	CYS 354, THR 320, PRO 286, TYR 308	MET 303, TYR 38, VAL 333, HIS 352
5.	kaempferol	-7.8	GLY 332, TYR 308, HIS 352, TRP 340, THR 320	MET 303, TYR 318, CYS 354
GRAM POSITIVE TRANSPEPTIDASE-PHENOLICS				
6.	Ellagic acid	-7.3	ASN 356, THR 320	TYR 318, TRP 340, HIS 352
7.	Syringic acid	-5.4	ARG 211, ILE 396	Nil
8.	Vanillic acid	-5.2	THR 220, HIS 214	VAL 196, LYS 217
GRAM POSITIVE TRANSPEPTIDASE-PHYTOSTEROL				
9.	Stigmasterol	-7.8	Nil	ILE 291, PHE 215, TRP 394, HIS 214
10.	Campesterol	-7.7	Nil	CYS 354, TYR 308, VAL 310, VAL 333

4.0 DISCUSSION

In this study, the induction of the various infectious diseases led to noticeable changes in all the parameters assessed. This includes raised body temperature of the rabbits due to the infections. The increased body temperature may be due to activation of cytokines such as interleukin-1, interleukin-2 and tumour necrosis factor-alpha which act as endogenos pyrogens (Wilson et. Al., 2011). Activation of the cytokines leads to a general increase in body temperature which depicts an infectious state (Gulati et. Al., 2016). Elevated body temperature may be due to compromised homeostatic function of the kidney due to infection. The observed appreciable decrease of pcked cell volume (PCV) during infection state when compared with with baseline may be due to degration of red blood cells during infection. The reduction may equally be due to infection of organs such as liver, spleen etc which infection may reduce their activities leading to reduced red cells productions. The loss of body weight may be due to excessive loss of body water i.e. diarrhea, which contribute substantially to body weight (Adekunle and Adekunle 2009).

Ingested bacteria may pass through the stomach and adhere to the epithelial cells lining the terminal small intestine,caecum and colon.

They enter the epithelial cells and penetrate into the underlying lamina propia, causing inflammation. It is the inflammatory response that mediates the release of prostaglandins which stimulates active fluid secretion, contributing to diarrhea.

Table 10. Gram negative transpeptidase-carotenoids

S/N	Ligands	Consensus Binding Affinity	Conventional Hydrogen Bond	Other bonds
	Clavulanic Acid (Standard)	-6.1	ARG 504, GLU 291	LYS 490
	Penicillin (Standard)	-7.5	SER 294, THR 487, GLY 543, SER 485	PHE 533
1.	Astaxanthin	-7.4	GLY 469	VAL 333, TYR 328, VAL 471, TYR 407, GLY 497, PHE 533
2.	Lycopene	-7.8	Nil	TYR 498, TYR 409, ARG 489, PHE 533, VAL 333
GRAM NEGATIVE TRANSPEPTIDASE-FLAVONOIDS				
3.	Isorhamnetin	-8.2	SER 294, ASN 351, SER 349, TYR 409, SER 485	VAL 333, ARG 489, PHE 533
4.	Epigallocatechin	-8.7	THR 329, ARG 331, TYR 532, SER 294, THR 487, TYR 498	TYR 409, VAL 333, ARG 333
5.	kaempferol	-8.1	SER 294, LYS 348, SER 349, THR 487, GLY 534, GLY 535	LEU 536, VAL 333, VAL 471, SER 485
GRAM NEGATIVE TRANSPEPTIDASE-PHENOLICS				
6.	Ellagic acid	-8.6	VAL 333, ASN 351, THR 487, GLY 535, GLY 534	SER 485, ASP 332
7.	Syringic acid	-6.0	GLY 535, GLY 534, THR 487	SER 485, SER 294, SER 349
8.	Vanillic acid	-5.6	GLY 177, ASN 283, PRO 384, ARG 387	ILE 287
GRAM NEGATIVE TRANSPEPTIDASE-PHYTOSTEROL				
9.	Campesterol	-8.3	THR 487	VAL 333, VAL 471, PHE 533, LEU 536
10.	Sitosterol	-8.3	Nil	LEU 536, VAL 333, ASN 351, VAL 471, PHE 533, PHE 472

Table 11. DHFR-Carotenoids

S/N	Ligands	Consensus Binding Affinity	Conventional Hydrogen Bond	Other bonds
	Trimethoprim (Standard)	-7.8	TYR 100, ALA 7, ASP 27	ILE 20, GLN 28
	Pyrimethamine (Standard)	-6.7	TYR 100, THR 46	ILE 20, ILE 5, ALA 7, PHE 31, ILE 94
1.	Astaxanthin	-7.9	ALA 7	PRO 58, LEU 57, ARG 32, LEU 50, ILE 20, PHE
2.	Lycopene	-6.8		ARG 44, ILE 14, ILE 20, LEU 82, ARG 67, LRU 65, ALA 126
DHFR-FLAVONOIDS				
3.	Isorhamnetin	-8.6	ASP 19, ARG 16, TYR 100, THR 46	ARG 45, ALA 126, GLY 96, ILE 20, ALA 7
4.	Biochanin	-7.9	ILE 5, ILE 94	PHE 31, GLN 28, SER 49
5.	Kaempferol	-8.5	TYR 100, THR 46, ASP 19, ARG 16, GLY 18	GLY 96, ALA 126, ARG 45, ILE 20, ILE 14, GLN 98
DHFR-PHENOLICS				
6.	Ferulic acid	-6.8	ILE 5, TYR 100, LEU 24, GLN 28	ILE 20, ILE 14, ALA 7
7.	Syringic acid	-7.4	ALA 7, ILE 94	PHE 31, GLN 28
8.	Vanillic acid	-6.6	GLN 28, TYR 100,	ALA 7, PHE 31, ILE 20
DHFR-PHYTOSTEROL				
9.	Ergosterol	-9.1	Nil	HIS 157, VAL 2, LEU 86, TYR 106
10.	Sitosterol	-9.1	Nil	GLU 33, LEU 153, ILE 34, HIS 30

Observed increased total protein during infection may be due to excessive loss of water through watery stool culminating in diarrhea. Total protein may high in patients with severe dehydration due to alteration in plasma volume. Increased total proteins may also results due to production of immunoglobulin dur to infection. Similar secretion of immunoglobulin fraction in response to infection might have led to the observed elevated globulin concentrations in the infectious state.

The administration of A. digitata ameliorated all the observed altered physical, biochemical and hematological parameters assessed. The observed efficacy of A. digitata in the treatment of the 3 induced infections may be due to presence of bioactive compounds which are of health benefits.

Several reports have shown medicinal benefits of vegetables which may include their antimicrobial activities (Sumathy et. Al., 2015).

Use of herbal medicines continues to be accepted due to the abundance of bioactive compounds with health benefits in some plants which have been reported to have antibacterial activities (Farahmandfar et. Al., 2019). In this study, characterization and identification of Phytochemicals in A. digitata showed presence of bioactive compounds including 30 flavonoids, 12 phenolics, 9 carotenoids and 5 phytosterols (Tables 4, 5, 6 and 7 respectively). Studies have reported

anticancer (Jia et al., 2020), bone protective (Zhang et. Al., 2022), antioxidative (Brown and Rice-Evans, 2015) activities of flavonoids. Phenolic compounds have been reported to exhibit antiviral, anti-obesity, antimicrobial, neuroprotective, cardioprotective and antioxidative activities (Muhammed et. Al., 2018). Carotenoids have been reported to express antioxidative, cardioprotective and eye protective activities (Sajilata et. Al., 2008). The phytosterols found in the medicinal plants are known to lower plasma total cholesterol and LDL-cholesterol levels. The cholesterol lowering effect may be due to direct inhibition of cholesterol absorption via brush border membrane (Melnikov, 2004), displacement of cholesterol from mixed micelles, and inhibition of endogenous cholesterol synthesis (Brauner et. Al., 2012). The observed efficacy of A. digitata in the treatment of the three induced infections may be due to abundance of identified secondary metabolites.

Although antimicrobial activities of A. digitata were confirmed by their ability to improve the altered physical, biochemical and hematological parameters, there is a need to understand possible mechanism of its antibiotic activities. This will entails evaluation of affinities of the phytochemicals for the target sites in the microbes via in-silico method/computational study. This will include proteins/enzymes critical for survival of the bacteria such as for DNA synthesis , mainteinance of cell wall integrity etc.

Virtual Screening Analysis

In this computational aspect, we used the binding affinities expressed by these ligands to predict the inhibitory activities against their target proteins (Gram+ and Gram- Transpeptidase and Dihydrofolate Reductase). It has been established that the *in silico* binding affinity expressed by a proposed drug candidate with its target receptor/protein could be estimated quantitatively as a measure of its ligand-receptor interaction (Pinzi and Rastelli, 2019). Fig1, Fig 2 and Fig 3 show the structures of Gram+-Transpeptidase, Gram-Transpeptidase and Dihydrofolate Reductase with PDB ID 6vs6, 6ile and 4gsu respectively used as target proteins for this study. Fifty four (54) compounds reported from our GC/MS results which belong to the group of phenolics (12), flavonoids (30), carotenoids (9) and phytosterols (5) were all docked to Transpeptidase (Gram+ and Gram-) and DHFR active sites where Calvulanic acid and Penicillin were both used as standards and the results are presented as Table 1, Table 2 and Table 3 respectively. For the Carotenoids-Gram+ Transpeptidase complex, astaxanthin and lycopene were selected as the best compounds with -8.3KJ/mol and -7.7KJ/mol binding affinities where astaxanthin forms two (2) hydrogen bonds (Thr308 and Gln244) with the active site while lycopene had no hydrogen bond interaction with the active site. For Flavonoids-Gram+ Transpeptidase, Isorhamnetin, Epigallocatechin and Kaempferol were chosen as the best candidate with binding affinities of -7.8KJ/mol, -8.2KJ/mol and -7.8KJ/mol respectively. Isorhamnetin formed two (2) hydrogen bonds (Thr320 and Trp340) with Gram+ Transpeptidase; epigallocathechin had four (4) hydrogen bonds (Cys354, Thr320, Pro286 and Tyr308) and kaemferol had five (5) hydrogen bonds (Gly332, Tyr308, His352, Trp340 and Thr320) with Gram+ Transpeptidase active site. Although, stigmasterol and campesterol have no hydrogen bond interaction, they exhibited good binding affinities of -7.7KJ/mol and -7.8KJ/mol respectively against the active site of Gram+Tranpeptidase. Comparing with the standards used (Calvulanic acid and Penicillin), our results show that epigallocatechin (-8.2KJ/mol) and kaemferol (-7.8KJ/mol) might stand out as the best prospective antibiotic not because of their best binding affinities but also, their high hydrogen bond interactions with the active site of the Gram+Transpeptidase.

The same carotenoids (Astaxanthin and Lycopene) were selected to have the interaction with Gram- Transpeptidase with astaxanthin having -7.4KJ/mol and lycopene having -7.8KJ/mol. Just as reported for the Gram+ Transpeptidase, astaxanthin has a single hydrogen bond (Gly469)interaction with the active site of Gram- Transpeptidase while lycopene has none. For the flavonoids, isorhamnetin, epigallocatechin and kaemferol interacted with Transpeptidase with -8.2KJ/mol, -8.7KJ/mol and -8.1KJ/mol respectively where isorhamnetin formed three (3) hydrogen bond interactions (Ser294, Asn351 and Ser349), epigallocatechin had six (6) interactions (Thr329, Arg331, Tyr4532, Ser294, Thr487, Tyr498) and kaemferol formed six (6) hydrogen bond interactions (Ser294, Lys348, Ser349, Thr487, Gly534 and Gly535) with Transpeptidase. Ellagic acid, syringic acid and vanillic acid interacted with Transpeptidase with binding affinity of -8.6KJ/mol, -6.0KJ/mol and -5.6KJ/mol respectively. Ellagic acid

had five (5) hydrogen bond interactions (Val333, Asn351, Thr487, Gly535, Gly534), syringic acid had three (3) interactions (Gly535, Gly534, Thr487) while vanillic acid expressed four (4) hydrogen bond interactions (Gly177, Asn283, Pro384, Arg387) with its target transpeptidase. The chosen flavonoids expressed the best binding affinities when compared with the two standards (Clavulanic acid (-6.1KJ/mol) and Penicillin (-7.5KJ/mol)) used. Besides these flavonoids, ellagic acid (-8.6KJ/mol) and lycopene (-7.8KJ/mol) also show better affinity when compared with the two standards. Campestrol and sitosterol interacted with the active site of Gram-Transpeptidase with the same binding affinity of -8.3KJ/mol while campesterol had only a single hydrogen bond (Thr487) with Gram- Transpeptidase. Ellagic acid (-8.6KJ/mol), kaemferol (-8.1KJ/mol), epigallocatechin (-8.7KJ/mol) and isorhamnetin (-8.2KJ/mol) both have better binding affinities and higher numbers of hydrogen bond interaction, meaning they could be a better antibiotics inhibitors of the Gram- Transpeptidase.

Dihydrofolate reductase (DHFR) was also docked against the three sets of compounds (Carotenoids, flavonoids and phenolics) where Trimothroprim and Pyrimethamine were both used as the standards and the result is presented as Table 3. Astaxanthin expressed a binding affinity of -7.8KJ/mol while lycopene showed -6.7KJ/mol with astaxanthin having three (3) hydrogen bond interactions (Tyr100, Ala7 and Asp27) while lycopene possessed two (2) (Tyr100 and Thr46). The flavonoids that expressed better binding affinity with DHFR were isorhamnetin (-8.6KJ/mol), biochanin (-7.9KJ/mol) and kaemferol (-8.5KJ/mol) with isorhamnetin having possessing four (4) hydrogen bonds (Asp19, Arg16, Tyr100 and Thr46) with DHFR, biochanin forms two (2) (Ile5, Ile94) while kaemferol had five (5) (Tyr100, Thr46, Asp19, Arg16, Gly18). Ferullic acid, syringic acid and vanillic acid binding affinities were -6.8KJ/mol, -7.4KJ/mol and -6.6KJ/mol with ferullic acid having four (4) hydrogen bond (Ile5, Tyr100, Leu24, Gln28), syringic acid possessing two (2) (Ala7 and Ile94) and vanillic acid having two (2) (Gln28 and Tyr100) interactions. Ergosterol and sitosterol interacted with DHFR with the same binding affinities of -9.1KJ/mol with no hydrogen bond interaction. Isorhamnetin (-8.6KJ/mol) and kaemferol (-8.5KJ/mol) were stronger inhibitors with better binding affinities and hydrogen bond interactions than the standards which are trimethoprim (-7.8KJ/mol) and pyrimethamine (-6.7KJ/mol). ADMET profile of selected compounds

The pharmacokinetic and pharmacodynamic assessment which involves Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) is a critical aspect of the early stage of drug discovery and delivery. It is meant to speed-up the discovery of hits and lead compounds into candidates for drug development. When the efficacy of drugs against their therapeutic target receptors is coupled with good ADMET attitude, it depicts high-quality candidate (de Souza et al., 2020 and Guan et al., 2018). Table 1 above shows the ADMET profile of the selected compounds using ADMETlab 2.0 web tool (Xiong et al., 2021). Selected compounds exhibited promising attitude of being absorbed in the human intestine (HIA) while all of them are not carcinogenic and non-hepatotoxic meaning they might be good drug candidates. Furthermore, they both have low and medium inhibitory capacity for CYTP3A4 which shows that their metabolism will not be inhibited. When drugs interact with HERG (Human ether a-go-go), it is being used as a biological marker/parameter to investigate the inhibitory potential of that drug against the myocardium potassium channels which must not be inhibited because it might lead to chronic heart challenges that could lead to death. All the selected phytochemicals do not inhibit HERG and these shows that they might be good drug candidates.

The biological modeling of drugs helps to predict the absorption capacity of prospective drug candidates using the Lipinski rule of 5 (RO5). The rule states that good absorption or permeation of a drug is more feasible if the chemical structure of the drug does not violate more than one of the following criteria/rules: (1) Molecular weight is less or equal to 500, (2) LogP should be less or equal to 5, (3) Hydrogen bond donor should be less or equal to 5, (4) Hydrogen bond acceptor should not be more than 10 (Lipinski, 2004). Besides the carotenoids that disobeys two of the rules (milogP and mw), all other selected compounds passed.

Table 12: Pharmacokinetic and pharmacodynamic (ADMET) properties of the bioactive compounds

Parameters		**Astaxanthin**	**Lycopene**		**Biochanin**	**Epigallocatechin**	**Isorhamnetin**	**kaemferol**		**Ellagic Acid**	**Ferullic Acid**	**Syringic Acid**	**Vanillic Acid**		**Campesterol**	**Stigmasterol**	**Sitosterol**	**Ergosterol**
Absorption																		
CaCo-2 (log cm/s)		-5.196	-5.708		-4.739	-6.306	-5.056	-4.739		-5.312	-4.902	-5.142	-5.159		-4.740	-4.668	-4.756	-4.747
P-Glycoprotein Inhibition		L	L		L	L	L	L		L	L	L	L		M	L	M	M
HIA		H	H		H	H	H	H		H	H	H	H		H	H	H	H
Bioavailability		>30	>30		>20	<20	>20	>20		>20	>20	>30	>20		>30	>30	>30	>20
Distribution																		
BBB Penetration		L	L		H	H	L	L		M	M	M	M		H	M	H	M
Metabolism																		
CYP1A2 inhibitor		L	L		L	L	H	H		L	L	L	L		L	L	L	L
CYP2C19 inhibitor	**CAROTENOIDS**	L	L	**FLAVONOIDS**	L	L	L	L	**PHENOLICS**	L	L	L	L	**PHYTOSTEROL**	L	L	L	L
CYP2C9 inhibitor		L	L		L	L	M	M		L	L	L	L		L	L	L	L
CYP2D6 inhibitor		L	L		L	L	M	H		L	L	L	L		L	L	L	L
CYP3A4 inhibitor		L	M		L	L	M	M		L	L	L	L		L	L	L	L
Excretion																		
Clearance (ml/min/kg)		0.719	-0.286		5.872	17.081	6.991	6.868		2.346	7.48	7.208	7.899		17.948	15.958	16.686	15.231
Half-time		0.067	0.137		0.754	0.87	0.922	0.905		0.863	0.926	0.946	0.941		0.015	0.014	0.013	0.028
Toxicity																		
hERG Blockers		L	L		L	L	L	L		L	L	L	L		L	L	L	L
DILI		L	L		M	L	H	H		H	M	H	H		L	L	L	L
AMES Toxicity		M	M		L	M	M	M		M	L	L	L		L	L	L	L
Carcinogenicity		L	L		L	L	L	L		M	M	L	L		L	L	L	L
Hepatotoxicty		M	M		L	L	L	L		M	M	L	L		L	L	L	L
Rat Acute Oral Toxicity		L	L		L	L	L	L		H	H	L	L		L	L	L	M

Table 13: Physicochemical properties of bioactive compounds

CAROTENOIDS					
Compound	**milogP**	**mw**	**nON**	**NOHNH**	**nviolations**
Lycopene	9.98	536.89	0	0	2
Astaxanthin	8.60	596.85	4	2	2
FLAVONOIDS					
Isorhamnetin	1.99	316.26	7	4	0
Kaemferol	2.17	286.24	6	4	0
Biochanin	2.80	284.27	5	2	0
Epigallocatechin	1.08	306.27	7	6	1
PHENOLIC					
VanillicAcid	1.19	168,154	4	2	0
SyringicAcid	1.20	198.17	5	2	0
FerullicAcid	1.25	194.19	4	2	0
Ellagic Acid	0.94	302.19	8	4	0
PHYTOSTEROL					
Campesterol	8.30	400.69	1	1	1
Ergosterol	7.18	396.66	1	1	1
Stigmasterol	7.87	412.70	1	1	1
Sitosterol	8.62	414.72	10	1	1

Conclusively, A. digitata is revealed to have effective antibiotic property against infections due to E. coli, Shigella flaxneri and Salmonella enteritis. The property was shown to be due to presence of secondary metabolites. Following the computational assessments, it can be observed that many of the identified phenolics, flavonoids, carotenoids and phytosterols may have antibiotic potentials, however, only a few can be proposed for possible testing clinically because of important factors outlined above. The study showed that antimicrobial acitivity of A. digitata involves inhibitions of gram +ve and gram _ve transpeptidase as well as dihydrofolate reductase. Following these, it can be proposed that the phenolics (vanillic acid, syringic acid, ferullic acid and ellagic acid), the carotenoids (Lycopene and astaxanthin), flavonoids (lycopene and astaxanthin), and phytosterols (campesterol, ergosterol, stigmasterol and sitosterol) compounds may be the best drug candidates as antibiotics against Salmonella enteritis, Shigella flaxneri and E.coli infections. More works are needed to actually determine the clinically relevance of the final phenolics, carotenoids, flavonoids and phytosterols. This may help in improving management of the different infectious diseases that are developing resistance to commonly use antibiotics.

Funding:

This study was self-funded.

Disclosure of interest

The authors report no conflict of interest

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