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Effects of *Salaha-A* (herbomineral drug) on blood parameters of rats and *in silico* inhibition of P-glycoprotein by it bioactive compounds

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The drug under study is a herbomineral drug containing tortoise shell ash and ground seeds of *Piper nigrum* used in the treatment of cancer. *Salaha-A* was found to contain essential minerals that could have medicinal importance to human. Calcium was found to be the major element in the tortoise shell ash and piperine is the pioneer alkaloid found in *Piper nigrum*. This work evaluates the effects of *Salaha-A* at different doses on blood parameters and spleen tissues of rats which result in significant increase in erythrocytes, lymphocytes and granulocytes. P-glycoprotein (P-gp) over expression is found in many types and many stages of cancer cells which impaired the delivery of anticancer drug to target site of action thereby leading to ineffective treatment. Hence, an inhibition of P-gp function is an attractive strategy toward multidrug resistance. It was further postulated that the conjugation of piperine and calcium can inhibit the function of P-gp, thus, molecular docking studies were carried out to predict 3D structure of P-gp and piperine-Ca conjugate. The *in silico* analysis shows higher binding affinity of piperine-Ca conjugate to P-gp model (-9.54 kcal/mol) in comparison with piperine alone (-8.77 kcal/mol). Piperine-Ca conjugate has shown good pharmacokinetic properties and therefore may be co-administered with anticancer drugs as efflux modulator after undergoing further *in-vitro* and *in-vivo* studies.

Key words: P-glycoprotein, herbomineral, calcium, *Piper nigrum*, piperine, pharmacokinetic.

INTRODUCTION

The medicinal use of tortoise/turtles shells has a very long tradition in the Asian countries. There are many reports related to the use of herbomineral therapeutics and were found to be one of the most promising areas of treating diseases like cancer (Sheikh et al., 2012). The drug under study, *Salaha-A*, is a combination of tortoise shell ash and powdered white pepper (*Piper nigrum* Linn.) at a ratio of 90:10, respectively. The powdered drug is administered orally with honey as vehicle, at a dose of 500 mg four times a day. It is locally prescribed to

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patients suffering from cancer and some degenerative diseases and described to possess anti-inflammatory and immunomodulatory effect. Herbal and mineral formulations possessing such a combination of preventive and therapeutic effects are described as *Rasayana* in ayurveda and have been widely used by ayurvedic physicians. The uses of herbs and minerals as integral parts of traditional systems of medicine are unique to the ayurvedic and Siddha systems of Indian Traditional Medicine (Kumar et al., 2006). These systems of medicine preparation obtained minerals from natural products following a series of purification and incineration.

Cancer has become one of the biggest challenges to the scientific community all over the world despite development of drugs and other modalities for it treatment. Cancer cells often become simultaneously resistant to multiple drugs. The molecular basis of multi drug resistance is the over expression of gene encoding P-glycoprotein (P-gp) which effectively extrudes hydrophobic drugs out of cancer cells, effectively precluding their activity (Ondieki et al., 2017). P-gp plays an important role in drug disposition and distribution. Several studies to enhance oral bioavailability have demonstrated the possible use of P-gp inhibitors that reverse P-gp-mediated efflux in an attempt to improve the efficiency of drug transport across the epithelia. P-gp inhibitor influences metabolism, absorption, distribution, and elimination of P-gp substrates in the process of modulating pharmacokinetics. Recently, *Salaha-A* is being prescribed by traditional medicine practitioners in Kano State, Nigeria and claimed to be effective in the treatment of cancer. Tortoise shell ash contains certain minerals with calcium being the most abundant. Several studies have shown that *P. nigrum* has antimicrobial (Dorman and Deans, 2000), antimutagenic (El-Hamss et al., 2003), antioxidant and radical scavenging property (Gulcin, 2005). Piperine being the main constituents of *P. nigrum* is known to exhibit a variety of biological activities and is anti-metastatic (Pradeep and Kuttan, 2002). Also, piperine has high immunomodulatory and antitumor activity (Sunila and Kuttan, 2004). Piperine also increases the bioavailability of certain drugs in the organism (Karan et al., 1999).

This study was therefore undertaken to ascertain the effect of *Salaha-A* at different doses on hematological parameters of rats. An examination of the histology of the spleen tissues of the rats given *Salaha-A* at different doses was also observed. Postulation of the conjugation of piperine with calcium can inhibit active functional site of P-gp using *in silico* analysis.

MATERIALS AND METHODS

Samples collection and handling

Pieces of tortoise shell were purchased from the local traditional medical center (Sangarib) packed in an airtight bottle, packed powdered white pepper (*P. nigrum* fruit) and bottle sealed pure natural honey were purchased from Jifatu store in Kano and were all stored in a dry place at room temperature. Twenty Wistar rats of both sexes weighing between 100 and 120 g (aged 6 to 7 weeks) were used in this study. They were procured from the animal house of Department of Biological Sciences, Bayero University, Kano. The rats were maintained in the animal room of the department. They were allowed to acclimatize for one week and fed on standard laboratory food pellets and water throughout the experiment.

Quantitative analysis of tortoise shell

Quantitative analysis of minerals like calcium, magnesium, iron, sodium, potassium and zinc of the tortoise shell ash was performed using Atomic Absorption Spectrometer (AAS). Six grams of the samples were weighed approximately in a 250 ml beaker; 50 cm^3 HCl solution (50% v/v) was added and kept for 1 h, then filtered to remove insoluble material. The samples were then transferred to a volumetric flask, volume adjusted to 50 ml and mixed. All precautions were taken to avoid contamination. The samples were then aspirated in atomic absorption spectrometer (AAS) against standard solution and the absorbance was measured.

Atomic Absorption spectrometer (AAS, mg/kg): Sample concentration × Volume made up / Weight of sample

Dosage schedule and drug administration

The dose for experimental animals was calculated by extrapolating the human dose (2000 mg/day) to animals based on the standard table of Paget and Barnes (1964). The therapeutic dose was calculated to be 180 mg/kg weight of rat. Honey was diluted with distilled water at a ratio of 2:3 in accordance with the guidelines for toxicity/safety profile evaluation of Bhasma/Raskalpas.

Conversion formula: Total clinical dose (a) × Conversion factor 0.018 (b) = (c) / 200 g weight of rat

2000 mg × 0.018= 36 mg/200 g weight of rat

36 × 1000/200 = 180 mg/kg

For the administration of the test drug at different doses, 20 rats were randomly assigned into four groups of five rats each. The first group served as control and were orally given 1 ml of honey solution. The remaining three experimental groups were orally given *Salaha-A* at 180, 280 and 380 mg/kg in 1 ml honey solution each for 15 consecutive days.

Determination of blood parameters

After the mentioned duration, the animals were weighed, sacrificed on 16th day and blood samples were withdrawn by cardiac collection into labeled EDTA bottles. For the complete blood count, the procedure followed was based on the instruction manual of Haematology Analyzer (Sysmex XP-300). Total red blood cell (RBC) count, hemoglobin content (HGB), haematocrit (HCT), granulocytes count (GR) total white blood cells (WBC), lymphocyte count (LYM), and platelet (PLT) were assessed. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet distribution width (PDW), mean platelet volume (MPV), and platelet larger cell ratio (P-LCR) were also calculated.

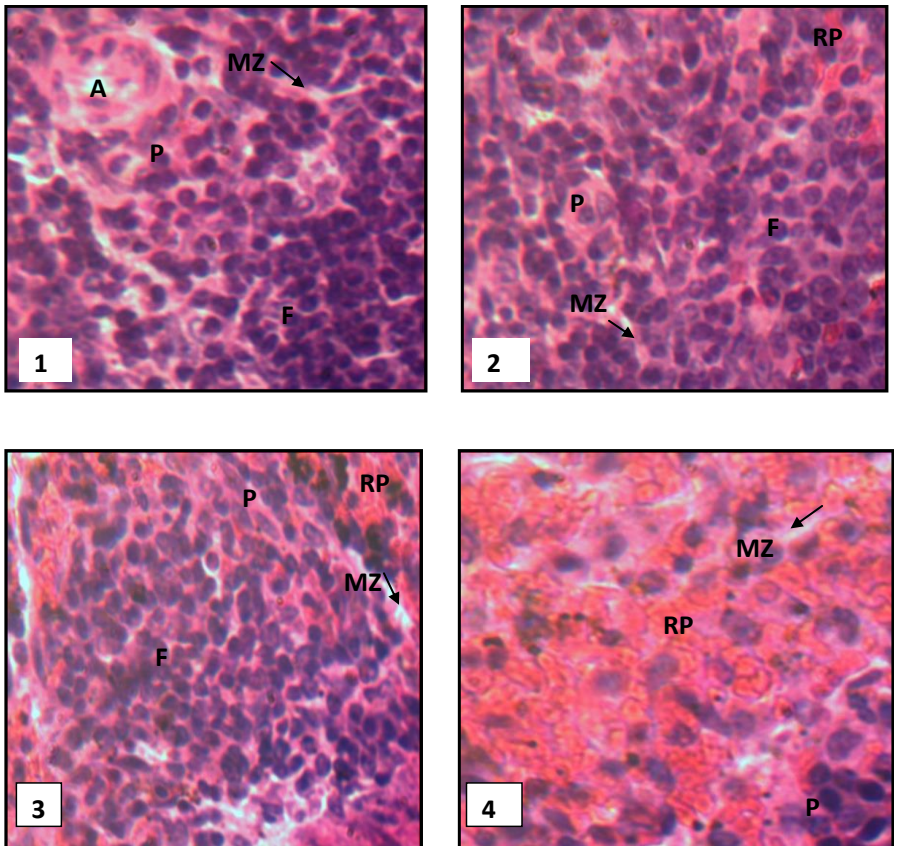

Figure 1. Histology of spleen tissues of rats given Salaha-A at different doses. A, Central artery; RP, Red pulp; MZ, Marginal zone; F, follicles; P; periarteriolar lymphoid sheath (PALS).

Determination of blood calcium level

Blood samples were centrifuged and by the method of Barnett et al. (1973), the resulting plasma was analyzed for total calcium using a colorimetric method (Randox, RX Monza CA kit no 590).

Reaction principle

Complex with O-Cresolphthalein complexone in an alkaline medium.

Sample material

Serum heparinized plasma diluted in 1+1 in 0.9% NaCl × 2

Concentration × 2.50 (mmol/l) = Sample / Standard

Histological examination of spleen tissues

The anterior abdominal wall muscle was incised to expose the gut in the abdomen. The spleen was carefully dissected out using a sharp sterilized scapel knife. The tissues were removed and were fixed in 10% formal saline in specimen bottles. Following fixation, the tissues were further processed by dehydration through ascending grades of alcohol. The first grade of alcohol used was 70% alcohol for a day followed by 90% alcohol overnight and finally two changes of absolute alcohol the following day. After dehydration, the tissues were treated with xylene (70% xylene/30% absolute alcohol) for a day followed by infiltration in three changes of paraffin at 60°C for two days, using an oven. Lastly, the tissues were transferred into an embedding medium (fresh paraffin wax) followed by blocking. Sections of about 5 microns thick were cut using a rotary microtome.

Haematoxylin and Eosin staining

Method of Drury and Wallington (1967) were adopted for Haematoxylin and Eosin staining. Sections were dewaxed for 2 min in each of the two changes of xylene and were transferred into absolute alcohol for removal of xylene for a minute and were stained with iron haematoxylin for 20 min. They were washed in running tap water for 2 to 3 min. Sections were differentiated in 1% acid alcohol for a few second, blued in running tap water for 5 min and counter-stained with 1% eosin for 3 min, then rinsed in water. They were dehydrated through ascending grades of alcohol (70, 90 and 100%) for 1 min each, cleared in xylene for a minute and mounted in distrene, plasticizer and xylene (DPX).

Photomicrography

Records of the histological results were obtained by photomicrography using a microscope with a camera at the Department of Anatomy, Bayero University Kano as shown in Figure 1.

Table 1. Minerals present in the tortoise shell ash.

Mineral	Weight (mg/kg)
Calcium	314.87
Iron	35.28
Magnesium	32.19
Sodium	3.09
Potassium	35.33
Zinc	20.34

Statistical analysis

Results are presented as mean ± standard error of mean (SEM) and total variation present in a set of data was analyzed through one way analysis of variance (ANOVA). Comparison between groups was done using least significant difference (LSD). Differences were considered statistically significant at $p<0.05$.

Homology modeling of P-glycoprotein (P-GP)

The sequence of P-glycoprotein (NP_001335874.1) was retrieved from National Center for Biotechnology Information (NCBI) and subjected to protein-protein Basic Local Alignment Search Tool (BLASTp) search against the Protein Database for the identification of a suitable template. Structures with higher identity, lowest E-value query coverage and good resolution (PDB ID 4Q9H, 4F4C) were selected as best templates. Multiple alignments of the target sequence and templates were generated by running the Advanced Modeling script in MODELLER 9v17 (Sali et al., 1995) based on a dynamic programming algorithm; it takes into account structural information from the templates when constructing an alignment. Five 3D structures were developed and the one with the low molpdf score was chosen as the best model. The developed model of P-gp was further evaluated by Ramachandran plot using Rampage server to assess the quality of the predicted model.

Binding site prediction

Binding site of the P-gp model was predicted by submitting the sequence to the COACH (Yang and Zhang, 2013) server. Residues from the group with the highest confidence score (C-score) and cluster size was selected as our functional active site for setting of x, y, and z grid size.

Piperine-calcium conjugate and retrieval of piperine

The structure of the piperine-Ca conjugate was drawn in the ACD-chemsketch software and then saved in sdf format. The sdf format of the piperine-Ca was converted to PDB and SMILES format using the Open BaBel Software (O'Boyle et al., 2011). The 3-Dimensional structure of piperine (PubChem CID: 638024) in sdf format was downloaded from PubChem database and also converted to PDB format in Open BaBel Software. The SMILES of the piperine-Ca conjugate and that of piperine obtained from PubChem Database were submitted to SwissADME server (Daina et al., 2017) for evaluation of their molecular properties and pharmacokinetic properties.

Molecular docking simulations

Docking simulations of piperine and piperine-Ca conjugate into the binding site of P-gp predicted model was performed using Autodock 4.2 (Norgan et al., 2011), the hydrogen polar atoms were added to the receptor molecules and Lamarckian algorithm was applied. Autogrid with a size of 60×60×60 points in X (-27.030), Y (28.281), and Z (-11.219) directions was built with a grid spacing of 0.375 Å.

RESULTS AND DISCUSSION

Quantitative analysis of tortoise shell ash

The quantitative analysis of the tortoise shell ash reveals that it contains minerals that are essential for maintenance of good health. Calcium, nutritionally important alkaline mineral was found to be the main active mineral found in the tortoise ash (Table 1).

Hematological parameters

The examination of blood gives the opportunity to investigate the presence of several metabolites and other constituents in the body of animals and it plays a vital role in the physiological nutrition and pathological status of an organism (Aderemi, 2004; Doyle, 2006). The significant increase of white blood cells (Table 2) in the rats indicates their capability of generating antibodies in the process of phagocytocis and have high degree of resistance to diseases (Soetan et al., 2013) and enhance adaptability to local environmental and disease prevalent conditions (Kabir et al., 2011; Iwuji and Herbert, 2012; Isaac et al., 2013). Lymphocytes are the main effectors cells of the immune system. They are formed and released from lymphoid tissue such as lymph nodes, spleen, etc. They are unable to eat or engulf organisms, but fulfill their function of defending the body against invading microorganisms, foreign macromolecules, and cancer cells (Junqueira and Carneiro, 2003).

Junqueira and Carneiro (2003) stated that in some cancers, e.g. melanoma and colorectal cancer, lymphocytes can migrate into and attack the tumor; this can sometimes lead to regression of the primary tumor.

The significant increase in red blood cells, haemoglobin and hematocrit, in all treated groups (Table 3) indicates the capacity of *Salaha-A* in the increase of delivering oxygen to the body tissues via blood flow through the circulatory system (Maton et al., 1997). The non-significant change in the Mean Platelets volume (MPV), Platelets distribution width (PDW) in therapeutic and high dose given groups indicates the reduction in production of platelets in the blood. The increase in proportion of whole blood occupied by platelets (PCT) in group that received the test drug at therapeutic dose was not highly significant. These results has showed the ability of Salaha-A in blood clotting was not achieved (Table 4).

In the adult human body, 99% of calcium is found in mineralized tissues (bones and teeth), in which it is present as calcium phosphate or calcium carbonate (Bedi et al., 2006). The remaining 1% is found in the blood, extracellular fluid, and various tissues. The maintenance

Table 2. Leukocytes indices of rats given at different doses of Salaha-A.

Parameter	WBC (×10^9/L)	LYM (%)	MID (%)	GR (%)
Control	3.38±0.1	53.08±0.5	25.16±0.9	10.8±0.68
180 mg/kg	3.52±0.4	58.4±0.7	28.05±0.5	13.3±0.3
280 mg/kg	5.8±0.5	70.9±0.64	23.2±0.64	6.11±0.2
380 mg/kg	3.94±0.35	86.2±0.8	11.51±0.44	2.99±0.28
LSD	0.93	1.75	1.62	1.05

Data represented as mean±SEM. LSD, Least significant difference.

Table 3. Erythrocytes indices of rats given Salaha-A at different doses.

Parameter	MCV (fL)	MCH (Pg)	MCHC (g/dl)	RBC (×10^{12}/L)	HGB (g/dL)	HCT (%)	RDW (%)	P-LCR (%)
Control	60.5±0.7	20.2±0.4	31.8±0.7	3.5±0.5	6.16±1.1	19.9±0.3	25.4±0.6	52.7±0.7
180 mg/kg	60.3±0.5	19.9±0.7	31.8±0.7	4.7±0.3	8.5±0.27	27.4±0.44	25.1±0.5	47.5±0.7
280 mg/kg	62.0±0.5	21.5±0.6	32.0±0.7	5.97±0.3	11.62±0.2	33.68±1.0	24.0±0.4	33.9±1.0
380 mg/kg	62.3±1.3	21.5±0.8	32.0±0.8	4.89±0.5	10.87±0.4	28.66±0.3	24.3±0.6	48.0±0.6
LSD	NS	NS	NS	1.15	1.8	1.07	NS	1.98

Data represented as mean±SEM, LSD, Least significance difference.

Table 4. Platelets indices of rats given at different doses of Salaha-A.

Parameter	MPV (FL)	PDW (%)	PCT (%)
Control	13.1±0.3	7.52±0.3	0.29±0.02
180 mg/kg	13.2±0.4	8.29±0.5	0.62±0.02
280 mg/kg	10.5±0.3	10.9±0.4	0.51±0.07
380 mg/kg	13.01±0.5	8.44±0.4	0.34±0.01
LSD	1.0	1.13	0.1

Data represented as mean±SEM. LSD, Least significant difference.

Table 5. Blood Ca^2 level at different doses.

Group	Ca^2 level (mmol/l)
Control	2.18±0.03
180 mg/kg	2.20±0.78
280 mg/kg	2.23±0.12
380 mg/kg	2.25±0.62

Data represented as mean±SEM.

of normal blood calcium level in rats given higher doses of Salaha-A (Table 5) may be achieved by well intestinal calcium absorption. The concentration of calcium in the blood, in which it is present as ionized calcium is maintained dynamically within a tightly regulated range through intestinal calcium absorption (Bedi et al., 2006). It was therefore concluded that the plant constituent of *Salaha-A* (Piperine) plays a significant role in enhancing the absorption of mineral contents of the test drug.

Histological results of spleen tissues

The spleen is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells (Cesta, 2006). It comprised 2 functionally and morphologically distinct compartments, the red pulp and the white pulp (periarteriolar lymphoid sheath, follicles, and marginal zone). The white pulp which surrounds the central arterioles contains lymphocytes and initiates immune responses to blood-borne antigens. The white pulp is abundantly seen in groups 2 (180 mg/kg) and 3 (280 mg/kg) and red pulp in group 4 that received the test drug at high dose of 380 mg/kg. The red pulp is anatomically well suited for its blood-filtering function and also contains macrophages that have special properties for fighting bacteria and facilitating iron metabolism (Mebius and Kraal, 2005).

In silico studies results

The multiple templates homology modeling of the P-gp resulted in five models and the best model among them was selected and evaluated using Ramachandran plot (Figure 2) with 96.3% in most favoured regions (1298 residues), 3.0% in allowed regions (40 residues) and only 0.7% in the outlier region (10 residues). The piperine-Ca conjugate was drawn in ACD-chemsketch (Figure 3) with molecular weight of 309.39 kcal/mol and the elemental analysis confirmed the composition of the piperine-Ca conjugate as $C_{13}H_{11}Ca_2NO_3$. The molecular docking simulation results (Figure 4) reveal Piperine-Ca to have

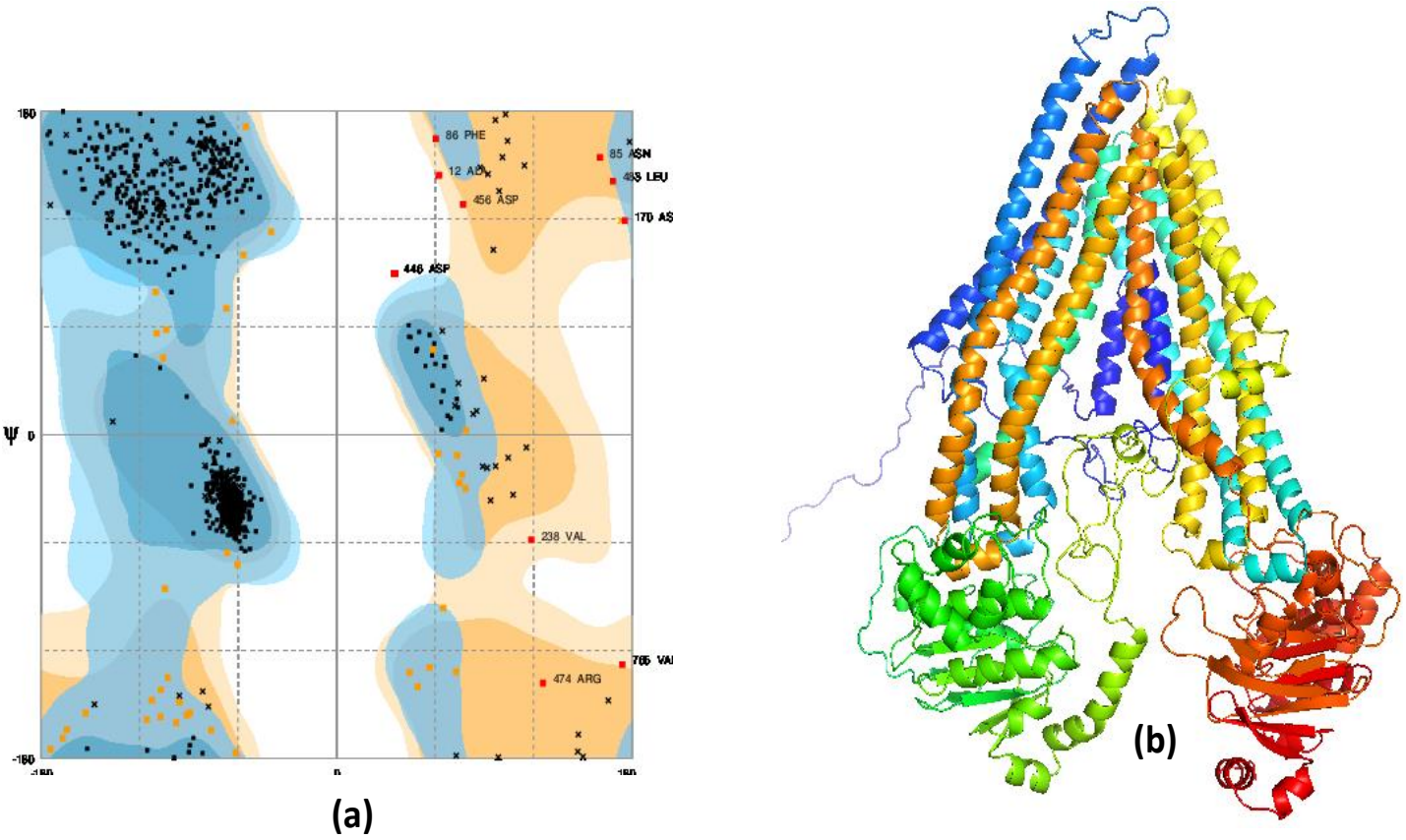

Figure 2. (a) Ramachandran plot showing distribution of residues in phi (Φ) and psi (ψ) torsion angles. **(b)** Predicted 3D structure of P-gp.

Table 6. Binding energy and pharmacokinetic properties of Piperine and Piperine-Ca.

Parameter	Piperine-Ca	Piperine
Binding energy	-9.54 kcal/mol	-8.77 kcal/mol
Molecular weight	309.39 g/mol	285.34 g/mol
WlogP	1.68	2.51
TPSA	38.77 Å²	38.77 Å²
LogS Ali	-2.98 (soluble)	-3.96 (soluble)
Log K_p (skin permeation)	-6.40 cm/s	-5.58 cm/s
GI Absorption	High	High
BBB Permeant	Yes	Yes
P-gp Substrate	No	No
Lipinkin's Rule of 5	Yes 0 violation	Yes 0 violation
Leadlikeness	Yes	Yes

WlogP, Water partition coefficient; TPSA, topological polar surface area; LogS, aqueous solubility; GI, gastrointestinal; BBB, blood brain barrier.

high binding affinity than piperine (Table 6). The piperine-Ca has better pharmacokinetic properties than piperine (Table 6).

Conclusion

From the outcome of the study, it could be concluded that *Salaha-A* contains compounds which have intrinsic importance to human health and could result in the increase of blood oxygen level. Conjugate of the bioactive compounds (piperine + calcium) could inhibit the function of P-glycoprotein and therefore can be efflux modulator that can be co-administered for the treatment of multidrug-resistant cancers.

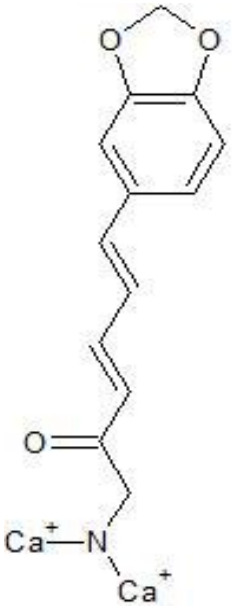

Figure 3. Piperine-Ca conjugates drawn using ACDchemsketch Software.

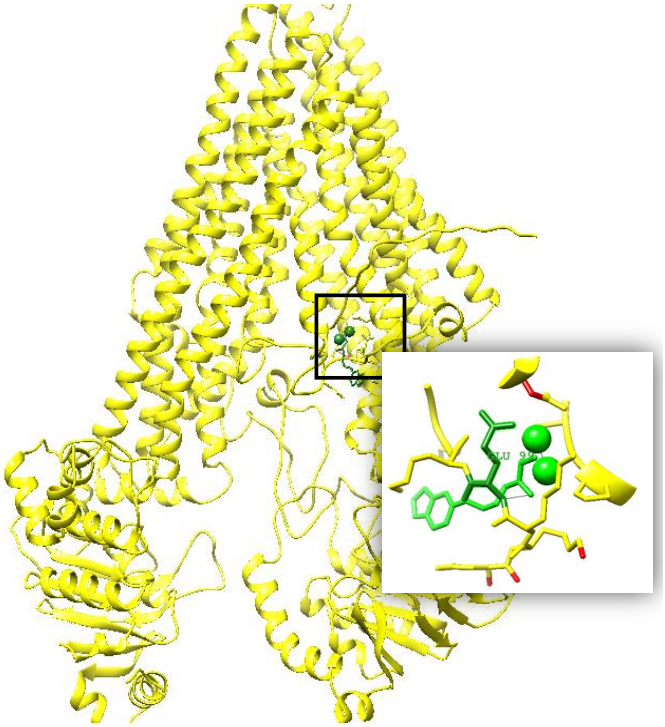

Figure 4. Piperine-calcium conjugate docked inside the binding site of P-gp.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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