

Evaluation of antileishmanial potential of *Gentiana kurroo* Royle by *in vitro* and *in silico* methods

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ABSTRACT

The objective of the present investigation was to evaluate the antileishmanial efficacy of *Gentiana kurroo* Royle by *in vitro* assay and *in silico* molecular docking method. *In vitro* antileishmanial screening of four different concentrations of the methanolic root extract of *G. kurroo* was carried out against promastigotes of *Leishmania donovani*. After *in vitro* evaluation, a total of 13 already known phyto-constituents present in the methanolic root extract of *G. kurroo* were docked *in silico* with five reported drug targets of *L. donovani* to evaluate their binding and inhibitory potential and to identify the active constituent responsible for antileishmanial activity. The methanolic extract of *G. kurroo* roots at a dose of 500 µg/ml showed 43.1% inhibition of *Leishmania* promastigotes after incubation for 24 hours at 25°C. *In silico* docking analysis of 13 phyto-constituents present in the roots of *G. kurroo* revealed that gentioside had maximum estimated binding and inhibitory capacity for most of the drug targets followed by norswertianolin. The findings of the present study suggested that these two compounds may be the major players responsible for the *in vitro* antileishmanial activity and therefore, should be further evaluated to establish their antileishmanial potential against amastigote and promastigote forms of *L. donovani*.

INTRODUCTION

Leishmaniasis is a major public health problem caused by an intracellular obligate protozoan parasite of the genus *Leishmania*. Leishmaniasis, by its various clinical forms, causes significant morbidity and mortality in the developing countries of the world every year. It is estimated that 0.2 to 0.4 million new cases of visceral leishmaniasis, 0.7 to 1.3 million new cases of cutaneous leishmaniasis with 20,000 to 30,000 deaths occur every year worldwide (Rama *et al.*, 2015). Despite the severity of this parasitic disease, till date, there is no vaccine available to efficiently prevent or cure leishmaniasis. The currently used chemotherapy has unpleasant side effects such as toxicity, variable efficiency

between different species and development of drug resistance in the parasite (Camacho *et al.*, 2003; Croft *et al.*, 2006). People living in rural areas of the developing countries are still dependent on traditional medicines to heal their ailments (Chan-Bacab and Pena-Rodriguez, 2001). There is a need to explore the potential of natural products obtained from the plants used by traditional healers which may eliminate the parasite from the host without causing any side effects. Several plant species have been screened for leishmanicidal activity after extraction with different solvents like petroleum ether, chloroform, hexane, ethyl acetate, methylene chloride, methanol and tested *in vitro* and *in vivo* against *Leishmania* parasite (Fournet *et al.*, 1996; Ghosh *et al.*, 2011; Pereira *et al.*, 2010).

The discovery and development of leishmanicidal biomolecules is also aided by the biochemical studies of the metabolic pathways of the parasite. In this approach, the enzymes essential for the survival of *Leishmania* are identified

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and targeted by lead molecules (Ogungbe and Setzer, 2013). Some known drug targets of *L. donovani* which are essential for the survival of the parasite and may be employed for *in silico* studies include adenylosuccinate lyase which converts adenylosuccinate to AMP and fumarate, an important step in purine salvage, Cyclophilin and *N*-myristoyl transferase are involved in co-/post-translational protein processing. Dihydroorotate dehydrogenase is involved in *de novo* pyrimidine biosynthetic pathway and pteridine reductase 1 is responsible for the salvage of pteridines (Ogungbe and Setzer, 2013; Boitz *et al.*, 2013; Sahi *et al.*, 2012).

Gentiana kurroo is a critically endangered medicinal plant belonging to the family Gentianaceae. It is mainly found in the North-Western Himalayas on south-facing steeper slopes along dry and rocky grasslands (Khuroo *et al.*, 2005). In traditional medicine, the roots and rhizomes of *G. kurroo* are used for the treatment of anorexia, bronchial asthma, gastric infections, leucoderma and urinary tract infections. It is also used as anthelmintic, astringent, antipsychotic, sedative, stomachic and carminative (Raina *et al.*, 2003).

The present study was aimed to assess the antileishmanial potential of methanolic root extract of *G. kurroo* *in vitro* against *L. donovani* followed by *in silico* docking of the reported phyto-constituents present in the methanolic root extract with five known drug targets of *L. donovani* to recognize the compound(s) responsible for the antileishmanial activity.

MATERIALS AND METHODS

In vitro assessment of antileishmanial activity

Preparation of extracts

Authenticated plants of *G. kurroo* were obtained from Plant Tissue Culture Laboratory, Department of Biotechnology, Shoolini University, Solan, H.P., India. The roots of the plants were thoroughly washed with tap water and subsequently with distilled water and air dried at room temperature. After complete drying, the roots were ground to yield a coarse powder. The powdered roots were extracted with methanol by Soxhlet extraction method for 24 hours. The extract was concentrated under reduced pressure using rotary evaporator. The dried extract was then stored in an air tight bottle at 4°C until use.

Parasite stock culture

The Axenic culture of *L. donovani* (LdMIPL-1) was maintained at 25°C in RPMI 1640 (Himedia) medium supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Himedia), streptomycin (150 µg/ml), penicillin G (100 µg/ml) and gentamycin (150 µg/ml) at pH 7.2.

Antileishmanial assay

For antileishmanial activity, promastigotes of *L. donovani* were sub-cultured in Schneider's Insect Medium (Himedia) supplemented with 10% heat inactivated FBS, streptomycin (150 µg/ml), penicillin G (100 µg/ml) and gentamycin (150 µg/ml). The antileishmanial screening

was performed in 96-well flat bottom tissue culture plates (Corning Life Sciences, USA). One hundred microliters of cell suspension containing 2×10^6 to 3×10^6 cells/ml was poured in each well of the plate. Four different concentrations of the methanolic root extract i.e. 100, 250, 350 and 500 µg/ml dissolved in dimethyl sulfoxide (< 0.025% v/v) were added to the culture. The plates were then incubated at 25°C for 24-48 hours. Sodium stibogluconate was used as positive controls and cell suspension with 0.025% DMSO was used as a negative control. The inhibition of the promastigotes was assessed by measuring the cleavage of 10 mg/ml of MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide} (Mossman, 1983). The absorbance was measured by using ELISA plate reader (BioTek, USA) at 595 nm. The percent growth inhibition was calculated by the following formula:

$$\% \text{ of inhibition} = \frac{\text{OD control} - \text{OD treated}}{\text{OD control}} \times 100$$

Statistical analysis

The antileishmanial assay was performed in triplicate with three replicates of each concentration tested. The results were expressed as mean \pm standard error of mean. The overall variation in a set of data was analysed by one way analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

In silico study

Selection and preparation of drug targets for docking

A total of five *L. donovani* proteins known as efficient drug targets were selected for docking (Table 1). Pdb files of 3D structures of all the drug targets were downloaded from Protein Data Bank and all the non-protein molecules (hetero-atoms) were removed. By using AutoDockTools-1.5.6, pdb files of all the receptors were modified by adding polar hydrogens and converted to pdbqt files which is an extended pdb format used for molecular docking analysis.

Table 1: List of *L. donovani* drug targets used for molecular docking analysis.

Drug target	Abbreviation	Accession number
<i>N</i> -myristoyl transferase	LdonNMT	PDB (2WUU)
Cyclophilin	LdonCyp	PDB (3EOV)
Dihydroorotate dehydrogenase	LdonDHODH	PDB (3C61)
Adenylosuccinate lyase	LdonASL	PDB (4MX2)
Pteridine reductase 1	LdonPTR1	PDB (2XOX)

Ligand preparation

A total of thirteen reported phytochemical constituents found in the roots of *G. kurroo* (Figure 1) were drawn using ChemDraw Ultra 10.0 software (Maurya *et al.*, 2012; Wani *et al.*, 2013). Smiles of all the structures were converted into pdb files using online smiles converter (<http://cactus.nci.nih.gov/services/translate/>). Pdb files were then optimised and converted to pdbqt files for molecular docking by using AutoDock Tools 1.5.6.

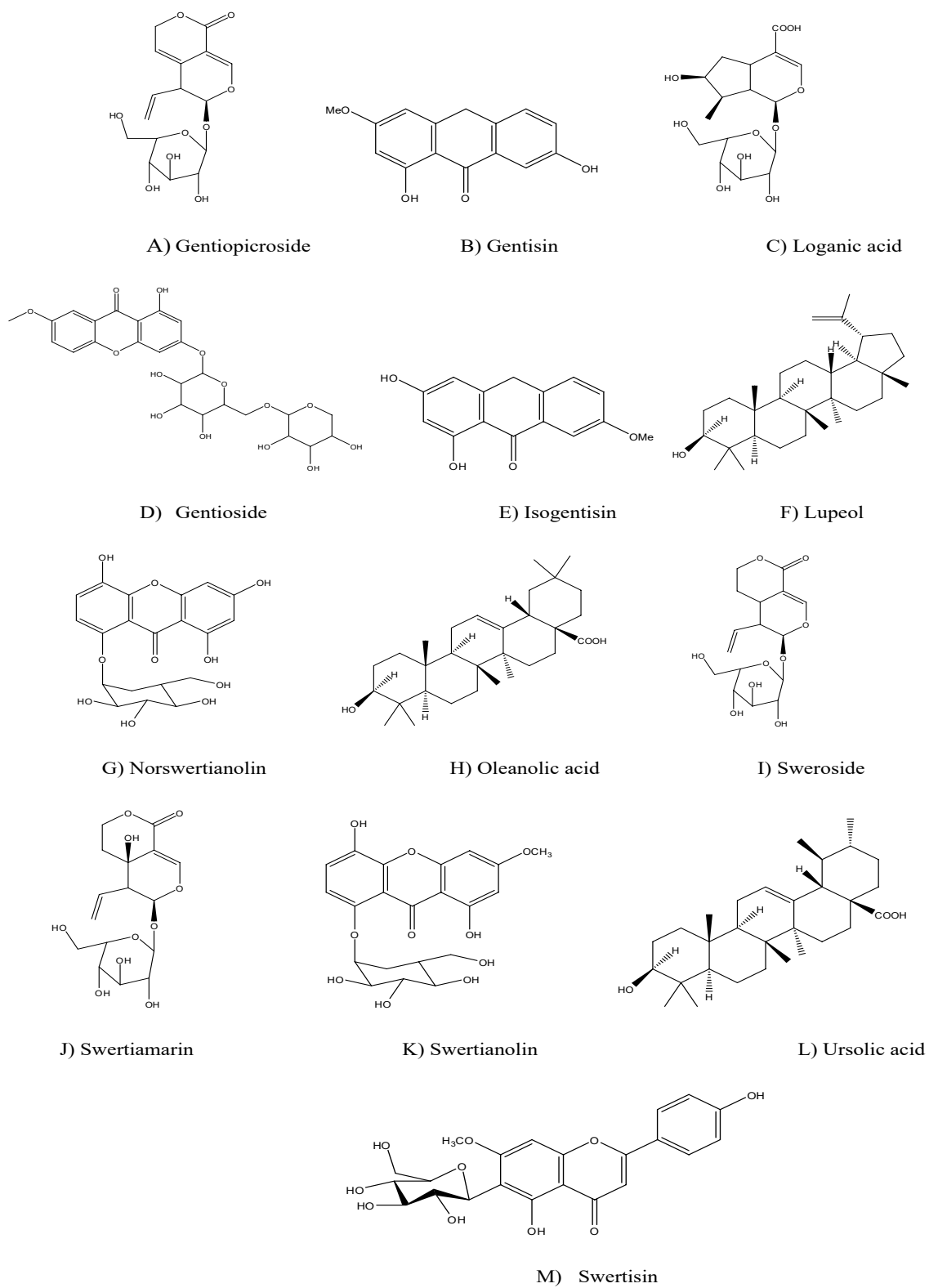


Fig. 1: Molecular structures of the phyto-constituents found in methanolic root extract of *G. kurroo* (A-M).

Molecular docking analysis

Molecular docking analysis was carried out to evaluate the efficacy of phyto-constituents found in the roots of *G. kurroo* against five drug targets which are essentially required for the survival of *L. donovani* by using AutoDock Vina with default parameters (Trott and Olson, 2010). The known active sites of various drug targets for inhibitors and substrates were identified using "Ligand Explorer" tool in Protein Data Bank (Barrack *et al.*, 2011; Brannigan *et al.*, 2010; Venugopal *et al.*, 2009).

RESULTS

In vitro antileishmanial activity

Methanolic root extract of *G. kurroo* was screened for antileishmanial activity against promastigote forms of *L. donovani*. The mortality rate of *L. donovani* was assessed by using MTT reduction assay. Maximum inhibition (43.1%) of the parasite was observed at the concentration of 500 µg/ml; followed by 41.68%, 40.49%, and 37.51% inhibition with concentrations of 350, 250 and 100 µg/ml, respectively (Figure 2). The antileishmanial activity of *G. kurroo* root extract was considerably low as compared to the sodium stibogluconate which was used as standard drug against *L. donovani* (Figure 3).

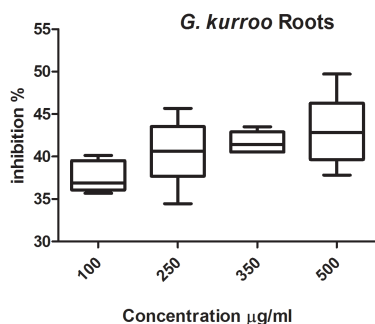


Fig. 2: Percent inhibition of the *L. donovani* promastigotes after 24 hours of incubation with four different concentrations of methanolic root extract of *G. kurroo*. The standard error of the mean was calculated as 0.731, 1.554, 0.502, and 1.685 in case of 100, 250, 350 and 500 µg/ml, respectively with a P value of 0.0279.

In silico docking analysis

In silico analysis by molecular docking of the phyto-constituents in the methanolic root extract of *G. kurroo* with five known protein targets of *L. donovani* was carried out to identify the compound(s) responsible for the leishmanicidal activity. The resultant receptor-ligand complexes were generated using PyMol Molecular Graphic System to know whether the ligands were bound to the amino acid residues of the active site or elsewhere in the receptor (Figure 4).

It was observed that all the phyto-constituents found in methanolic root extract of *G. kurroo* have considerably low binding energies against all the five *L. donovani* drug targets (Table 2). Gentioside and norswertianolin exhibited minimum binding energies with most of the drug targets used. Out of these two only gentioside was bound to the active site of the four protein targets i.e. LdonASL, LdonCyp, LdonNMT and LdonPTR1.

Norswertianolin was bound only to the active site of LdonDHODH whereas; the binding was random for the other four receptors and somewhat away from the active site. The active site amino acid residues of all the five drug targets to which the best ligands were bound are enlisted in Table 3. LdonASL and LdonNMT are the two proteins which have shown very low binding energies with most of the phyto-constituents but their active sites were properly blocked only by gentioside. The binding modes of each ligand may be different for all the drug targets used as the active sites of all the targets possess characteristic amino acid residues which make them unique. Therefore, the gentioside was interacted with different amino acid residues in all the drug targets (Figure 4a, 4b, 4d, 4e). The *in silico* study revealed that gentioside may be responsible for the antileishmanial activity shown by the methanolic root extract of *G. kurroo*.

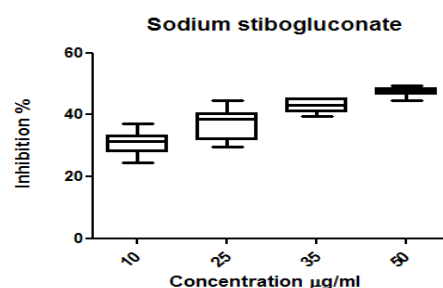


Fig. 3: Percent inhibition of the *L. donovani* promastigotes after 24 hours of incubation with four different concentrations (1/10th of *G. kurroo* extract) of standard drug (sodium stibogluconate).

DISCUSSION

Leishmaniasis, being endemic in developing countries, establishes a worldwide health problem. Control of the disease becomes more difficult due to lack of an effective vaccine and some safe chemotherapeutic agents. The current chemotherapy of leishmaniasis includes pentavalent antimonials, liposomal formulations of amphotericin B, pentamidine and miltefosine and all of these are associated with one or more drawbacks which limit their use (Santos *et al.*, 2008). In the search of some new leishmaniasis control agent research is going on worldwide and is more concentrated towards the use of natural products rather than synthetic chemicals. Biological molecules extracted from plant and marine natural products represent an important source of novel therapeutics against various forms of leishmaniasis (Rocha *et al.*, 2005; Tempone *et al.*, 2011).

G. kurroo is used in ethno-medicine for the treatment of several diseases since ancient times. The methanolic root extract of *G. kurroo* is known to have potential anticancer activity by inducing apoptosis in human pancreatic cancer cell line (MiaPaCa-2) through the disruption of mitochondrial membrane potential (Wani *et al.*, 2013). Lupeol and ursolic acid which are found in the methanolic root extract of *G. kurroo* possess significant immune-modulatory activity (Maurya *et al.*, 2012). Methanolic whole plant extract has been reported to possess anti-inflammatory and immunosuppressant activities (Khan *et al.*, 2014). *G. kurroo* has not been studied so far for its antileishmanial activity.

In the present study methanolic extract of *G. kurroo* roots was evaluated for its antileishmanial activity against *L. donovani*.

We have observed 43.1% inhibition with a concentration of 500 µg/ml. The findings of the study are in accordance with the previously reported studies on *Artemisia aucheri* and *Camellia sinensis* where the methanolic extracts of these plants at concentrations of 600–750 µg/ml showed significant antileishmanial activity against both amastigote and promastigote forms of *L. major* (Sharif *et al.*, 2006). In another study, it was reported that methanolic extract

of *Alstonia scholaris* at a concentration of 500 µg/ml showed an inhibition of 40.3% inhibition of *L. donovani* promastigotes (Sidana and Farooq, 2015). The activity shown by methanolic extract of *G. kurroo* in the present study was considered significant as the plants used for extraction were *in vitro* cultivated and were not obtained from their natural habitat.

Table 2: Binding energies of the 13 phyto-constituents found in the methanolic root extract of *G. kurroo* against five *L. donovani* drug targets (kcal/mol).

Receptors -> Ligands	LdonASL	LdonCyp	LdonDHODH	LdonNMT	LdonPTR1
Gentiopicroside	-8.1	-7.0	-6.8	-8.4	-8.5
Gentioside	-10.4	-8.8	-8.0	-10.3	-8.9
Gentisin	-8.3	-7.4	-7.3	-9.7	-8.0
Isogentisin	-8.6	-7.7	-8.0	-9.7	-8.2
Loganic acid	-7.8	-7.3	-7.0	-8.9	-8.0
Lupeol	-9.4	-6.6	-7.6	-9.3	-7.4
Norswertianolin	-8.8	-8.1	-9.6	-10.4	-8.6
Oleanolic acid	-10.2	-6.6	-8.2	-9.7	-7.6
Sweroside	-8.6	-7.2	-7.0	-8.6	-7.8
Swertiamarin	-8.7	-7.0	-9.3	-8.0	-7.6
Swertianolin	-9.2	-8.2	-7.5	-10.1	-8.7
Swertisin	-9.4	-7.2	-7.3	-10.2	-7.5
Ursolic acid	-9.3	-7.0	-8.0	-9.8	-7.6

Table 3: List of amino acid residues of the active sites of drug targets to which the used ligands were bound.

Serial Number	Drug Target	Amino acid residues in active sites
1	<i>N</i> -myristoyl transferase	Leu169, Val171
2	Cyclophilin	Arg78, Gln86
3	Dihydroorotate dehydrogenase	Leu72, Lys 44, Asn195, Ser 196, Asn68, Gly272
4	Adenylosuccinate lyase	Gln-273, Thr-148, Ser-149
5	Pteridine reductase 1	Ser40

In earlier studies, thirteen phyto-constituents have been reported in the methanolic root extract of *G. kurroo*. These suspected phyto-constituents must be validated and voted up for further *in vitro* and *in vivo* antileishmanial assays to minimize the expenses and time consumption. This may be achieved by molecular docking analysis of the phyto-constituents with potential drug targets. Virtual screening is a useful method for the preliminary screening of the drugs against a particular pathogen as it requires less time and resources. It is extensively used for screening of synthetic compounds as well as natural products against *Echinococcus*, *Plasmodium*, *Mycobacterium* and other tropical infectious agents (Crowther *et al.*, 2010; Mehlin, 2005; Prakash *et al.*, 2010; Sundaramurthi *et al.*, 2011).

In this study, we have targeted five *L. donovani* drug targets for the preliminary screening of thirteen reported phyto-constituents by *in silico* protein-ligand docking to find out the compound responsible for *in vitro* antileishmanial activity and to validate it for further experiments. In molecular docking, we observed that gentioside was able to block the active sites of four out of five drug targets used and may play an important role in the inhibition of their catalytic activity which may lead to interruption

of various important metabolic pathways of *L. donovani*. On the other hand, norswertianolin, which has shown minimum binding energy for LdonDHODH and blocked its active site, may cause interruption in *de novo* pyrimidine biosynthesis in the parasite thereby inhibiting the parasite growth and multiplication. The remaining eleven compounds have shown low binding energies with most of the drug targets used; however, those compounds were binding to the sites away from the known active sites of the drug targets.

CONCLUSION

The findings of the present study suggested that methanolic root extract of *G. kurroo* possess significant antileishmanial activity against the *L. donovani* promastigotes. *In silico* docking revealed that both gentioside and norswertianolin may be the major players responsible for the *in vitro* antileishmanial activity of methanolic root extract of *G. kurroo*. These two compounds have never been tested before for their antileishmanial potential. Thus, further studies are directed at purification of these two compounds and their screening for antileishmanial activity against amastigote and promastigote forms of *L. donovani*.

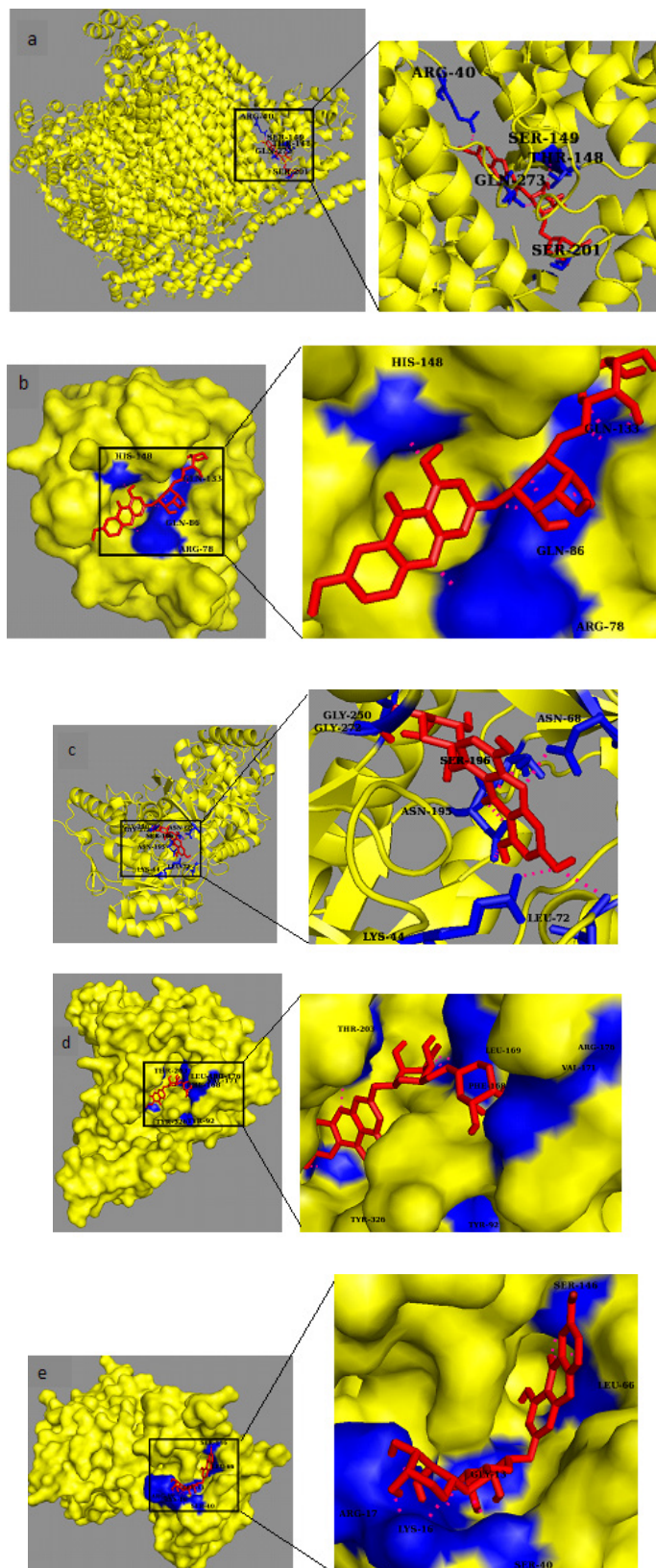


Fig. 4: Ligand-receptor complex models of best docked ligands (phyto-constituents in root extract of *G. kurroo*) showing the interaction of ligands in the known active sites of five receptors (a) LdonASL with Gentioside, (b) LdonCyp with gentioside, (c) LdonDHODH with norswertianolin, (d) LdonNMT with gentioside and (e) LdonPTR1 with gentioside. Here, ligand is shown in red, yellow is the surface of drug target, blue are the active amino acid residues to which the ligand is bound and pink dotted lines are the hydrogen bonds between ligand and amino acid residues.

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