Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2020; 9(1): 1260-1266
Received: 16-11-2019
Accepted: 18-12-2019

Ranjith D
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala, India

Surjith KP
Assistant Professor, Department of Veterinary Anatomy, College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala, India

Corresponding Author:
Ranjith D
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala, India

In silico anthelminthic activity of a novel herbal formulation

Ranjith D and Surjith KP

Abstract
Helminth parasitism, is one of the major health problems severely limiting the animal productivity in dairy animals. In spite of significant production losses, which may run into millions of rupees the problem is neglected due to its chronic and insidious nature. The diverse agro climatic conditions, animal husbandry practices and pasture management largely determines the incidence and severity of various parasitic diseases in a region. Even though many of the modern drugs are effective as anthelmintics, many parasitic helminthes of veterinary importance have genetic features that favor development of anthelmintic resistance, this becoming a major worldwide constrain in livestock production. The strategy is slowly shifting towards the use of herbal anthelmintics. In the present study, an *In silico* study was designed to disclose the anthelminthic property of a novel herbal formulation having rhizomes of curcuma longa and cassia alata respectively. The major phytoconstituents of the formulation viz.. curcumin, demethoxy curcumin, bis demethoxy curcumin, aloe emodin and kaempferol were screened for anthelminthic acidity against fumarate reductase enzyme with flutolanil as standard drug. All the phytoconstituents depicted good inhibition against the enzyme more than the standard drug, among which curcumin emerged as potent inhibitor of the enzyme with binding energy -9.49 kcal/mol followed by aloe emodin with -9.13 kcal/mol and bis demethoxy curcumin with -9.07 respectively. In conclusion, the present herbal formulation can be used as potent herbal anthelmintics.

Keywords: Helminths, curcuma longa, cassia alata, curcumin, aloe emodin, autodock

Introduction
Livestock plays an important role in Indian economy and is an important subsector of Indian Agriculture. Among the livestock population, cattle plays a major role in India's economy, accounting 16.24% of world bovine population (Livestock census, 2012) [1]. Gastrointestinal (G.I.) parasitic infections are common in dairy cattle causing considerable economic losses as a consequence of mortality in infected animals and reduced weight gain. It is a worldwide problem for both small and large scale farmers and is a great threat to livestock industry (Saddiqi *et al*., 2010) [2]. It is recognized as a major constraint to production by causing clinical and subclinical parasitism. Subclinical G.I. parasitic infections are most common and economically important in cattle in India (Chowdhury & Tada, 1994) [3]. Most of the economic losses are due to subclinical effects which go unnoticed to the owner's inspite of frequent contact. The economic losses caused by gastrointestinal parasites are multifarious: lowered fertility, reduced work capacity, reduction in food efficiency and lower weight gain, lower milk production, increased treatment cost and mortality in heavily parasitized animals (Fikru *et al*., 2006) [4]. The effects of helminths infection on the physiology of the host animal as a result of a specific host/parasite combination, are highly dependable upon the size of the infectious dose, the predilection sites of the parasite and the population density at these sites combined with its ability to evade the immune response by the host. Moreover, the physiological impact of the infection can directly or indirectly be influenced by the presence of other infectious agents such as other helminths, protozoans and/or various microbes. The immunopathological interaction between these agents are only partly understood and the attempt to explain a more than additive effect of combined infections in pathophysiological and/or energetical terms has not been successful (Over *et al*., 2020) [5]

The negative impacts of helminths on livestock productivity still remain a major challenge in the livestock industry globally (Wilson, 2011) [6] despite the projected increased dependence on agriculture in the nearest future (Herrero & Thornton, 2013) [7]. These parasites cause serious economic losses in ruminants ranging from growth rate decrease and poor quality of skin and hides to reductions in the production of milk, meat and wool (Qamar *et al*., 2011) [8]. For instance, evidence revealed that lactating cows may lose 294.8 kg of milk on average per lactation due to helminths parasites (Ploeger *et al*., 1990 & Nodtvedt *et al*., 2002) [9, 10]. Economic losses caused by the rejection of editable organs of slaughtered food animals during

veterinary meat inspections were also documented (Biu *et al*., 2006, Danbirni *et al*., 2015 & Karshima *et al*., 2016a) [11-13]. Despite these benefits, helminths infection still cause serious economic losses as a result of reductions in milk production, weight gain, fertility and carcass quality (Karshima *et al*., 2018) [14]

The significance of Helminthiasis has been recognized by livestock farmer's right from the earliest of times and various methods have been employed by them to control helminths in their animals including the use of medicinal plants and herbs and different grazing techniques (Bukhari & Sanyal, 2011) [15].

The use of plants as medicine has long being in existence and are widely documented in records kept in china, India and Egypt. Undoubtedly these ancient practices were discovered by series of "trial and error", which then could not be substantiated by proven scientific theories. But from last few decades, there has been global resurgence of interest in indigenous knowledge of herbal drugs due to various reasons. Medicinal plants may become good alternatives for modern synthetic anthelminthic in developing countries particularly in small farms if their efficacy is proved scientifically under control studies. A number of plant materials have been tested for their anthelminthic activity and found effective against many parasites (Agnihotri RK, 2020) [16].

The present study explores the traditional use of herbal formulation for helminthiasis, consist of equal proportion of rhizomes of Turmeric (*curcuma longa*) and cassia alata. Turmeric has been investigated widely and is said to exhibit different properties such as anti-inflammatory, hypercholesterolemic, anthelminthic, choleretic, antimicrobial, insect repellent, antirheumtaic, antifibrotic, antivenomous, antiviral, antidiabetic, and antihepatotoxic as well as anticancerous properties (Akram *et al*., 2010 and Velayudhan *et al*., 2012, Nasai *et al*, 2016) [17-19]. Curcumin, demethoxy curcumin and bisdemethoxy curcumin collectively known as curcuminoids (3-6%) are major polyphenolic compounds in turmeric rhizomes (Ravindranath and Satyanarayan 1980, Satyawati *et al* 1976, Abhishek and Dhan Prakash, 2008) [20-22].

Pharmacological investigations performed so far on cassia alata have shown that this herb has several biological activities, such as antimicrobial (Ibrahim and Osman 1995; Somchit *et al*. 2003) [23, 24], antifungal (Damodaran and Venkataraman 1994; Villaseñor *et al*. 2002) [25, 26], anti-inflammatory (Moriyama *et al*. 2003) [27], purgative, as an expectorant, as an astringent and as a mouthwash (Quisumbing 1978; Rai 1978) [28, 29], analgesic (Palanichamy and Nagarajan 1990) [30], and antitumor (Belkin *et al*. 1952) [31] activities and anthelminthic activity (Suman *et al*., 2012) [32]

NADH-fumarate reductase is part of a unique respiratory system in parasitic helminths (Boveris *et al*., 1986, Takamiya *et al*., 1994, Van *et al*., 1994, Fioravanti *et al*., 1998) [33-36] and is the terminal step of the phosphor-*enol*-pyruvate carboxykinase - succinate pathway, which is found in many anaerobic organisms (Saz, 1981, Bennet *et al*., 1988, Marr *et al*., 1995) [37-39]. The composition and linear sequential order of the respiratory components of NADH-fumarate reductase have been elucidated with mitochondria from the parasitic nematode, *Ascaris suum* (Kita *et al*., 1997, Kuramochi *et al*., 1994, Saruta *et al*., 1995, Amino *et al*., 2000) [40-43]. Electrons from NADH are accepted by rhodoquinone through complex I (NADH-rhodoquinone oxidoreductase) and then transferred to fumarate through complex II (rhodoquinol-fumarate reductase). This anaerobic electron transport couples site I phosphorylation in complex I by translocating protons across the inner mitochondrial membrane, providing ATP even in the absence of oxygen. Although this system has been thought to be a good target for developing anthelmintics, only a few compounds have been reported [e.g., bithionol (Ikuma *et al*., 1993) [44] and thiabendazole (Kohler *et al*.,1978) [45] to be inhibitors of rhodoquinol-fumarate reductase, and the relationship between their enzyme inhibitions and anthelmintic activities is not clear because their inhibitory activities are weak.

Therefore the present study explores the *In silico* anthelminthic property of the major phytoconstituents from the herbal formulation viz.. curcumin, demethoxy curcumin, bisdemethoxy curcumin, aloe emodin and kaempferol against fumarate reductase enzyme.

Materials and Methods

Selection of protein and preparation of its structure

The docking studies was carried out by taking X-ray crystal structure data to understand the molecular interactions of the phytocompounds of the herbal formulation with binding site of the selective protein. Mitochondrial Fumerate Reductase (PDB ID: 3VRB) were evaluated in the present study. The protein structures were downloaded from protein data bank (http://www.rcsb.org/pdb/) established by Brookhaven national laboratory (BNL) in 1971 (Sheela Devi *et al*., 2015) [46] necessary hydrogen atoms were added along with Gasteiger- Marsili charges (Begum, 2017, Paarakh, 2017, Anagha, 2016) [47-49]. All the solvent molecules and co-crystallized ligands were removed from the structures in order to use as a receptor for docking (Suganya and Radha Mahendran, 2016) [50] by removing the water not involved in ligand binding and ligand molecules, inserting missing atoms and correcting the valancies (Priyanka James *et al*., 2017) [51].

Active Site

The active site is predicted using PDBsum, which is a pictorial database of 3D structures in the Protein Data Bank database. The default active site were considered of docked complexes, Amino acid within 10 ^{0}A by cogitating ligand of interest in center (Rekha and Chandrashekhara, 2017) [52].

Selection of ligand and preparation of its structure

A total of five ligands of both *cassia alata* and *curcuma longa* rhizomes were selected by literature survey includes kaemferol (Pub chem ID: 5280863), aloe emodin (Pub chem ID: 10207), curcumin (Pub chem ID: 969516), demethoxy curcumin (Pub chem ID: 5469424) and bisdemethoxy curcumin (Pub chem ID: 5315472) respectively. The ligands were drawn in Chem Draw Ultra 6.0 (Chem Office package) assigned with proper 2D orientation and the structure of each compound was analyzed for connection error in bond order.

In silico molecular Docking studies:

The molecular docking study was used to understand the possible best binding pose of the compounds by which they could be sorted for identifying promising leads using Autodock 4.2.6 respectively (Alam *et al*., 2016) [53]. The enzyme Fumerate reductase was retrieved from RCSB; Protein data bank (PDB ID: 3VRB) respectively. The energy of the molecules were minimized using Swiss PDB viewer, the energy minimized compound were then read as input molecule for Auto Dock 4.2.6 in order to carry out docking simulation studies (Schuttelkopf and Aalten, 2004 & Morris

et al., 1998) [54,55]. All the heteroatoms were removed from 3vrb.pdb, to make complex receptor free of any ligand before docking. The Graphical User Interface program "Auto- Dock Tools" was used to prepare, run, and analyze the docking simulations. The unwanted water molecules were also removed from the grid. The only chain A was selected in the refine tab. Kollman united atom charges, solvation parameters and polar hydrogen's were added to the receptor for the preparation of protein in docking simulation. Since ligands are not peptides, Gasteiger charge was assigned and then non-polar hydrogens were merged. AutoDock requires pre-calculated grid maps, one for each atom type, present in the ligand being docked as it stores the potential energy arising from the interaction with macromolecule. This grid must surround the region of interest (active site) in the macromolecule. Docking software AutoDock 4.2.6 program supplied with Auto Grid 4.0 and Auto Dock 4.0 was used to produce grid maps. The spacing between grid points was 0.375 angstroms. The Lamarckian Genetic Algorithm (LGA) was chosen to search for the best conformers. During the docking process, a maximum of 10 conformers was considered for each compound. All the Auto Dock docking runs were performed in Intel (R) Core (TM) i5-7200U-CPU @ 2.50GHz 2.71GHz, with 8 GB DDR2 RAM and 64 bit Operating System, x64 – based processor. Auto Dock 4.2.6 was compiled and run under Microsoft Windows 10 pro operating system. Docking scores are used to predict binding modes, binding affinity, and orientation of synthetic ligands at the active site of protein.

Results

Table 1: Molecular docking results of target molecules with 3VRB:

Sl. No	Ligands	3VRB						
		B.E (kcal/mol)	I.C (µM)	In. E (kcal/mol)	I.E (kcal/mol)	T.E (kcal/mol)	U.E.E (kcal/mol)	R.RMS (kcal/mol)
1	Aloe emodin	-9.13	203.5	-10.32	-1.28	1.19	-1.28	89.03
2	Kaemferol	-8.54	546.4	-10.04	-1.53	1.49	-1.53	86.91
3	Curcumin	-9.49	110.41	-12.47	-1.7	2.98	-1.7	85.78
4	Demethoxy curcumin	-8.94	278.12	-11.63	-1.01	2.68	-1.01	85.42
5	Bisdemethoxy curcumin	-9.07	224.98	-11.46	-0.51	2.39	-0.51	86.67
6	Flutolanil	-6.68	12.66	-8.17	-0.64	1.49	-0.64	86.77

B.E – Binding Energy, I.C – Inhibition Constant, In. E – Intermolecular Energy, I.E – Internal Energy, T.E – Torsional Energy, U.E.E – Unbound Extended Energy, R.RMS – Reference Root Mean Square

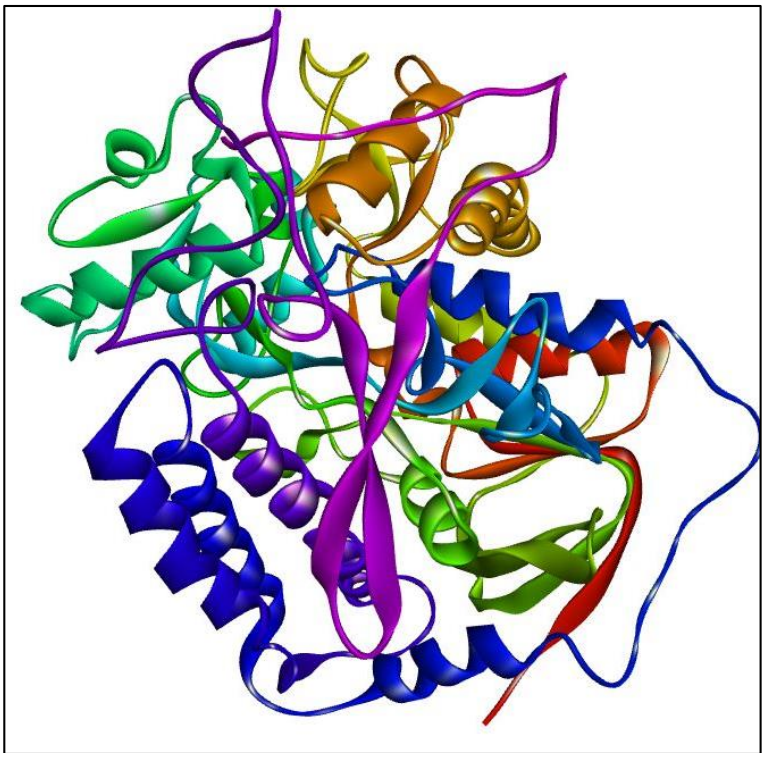

Fig 1: X Ray Crystallographic Structure of Fumarate Reductase enzyme (PDBID: 3VRB)

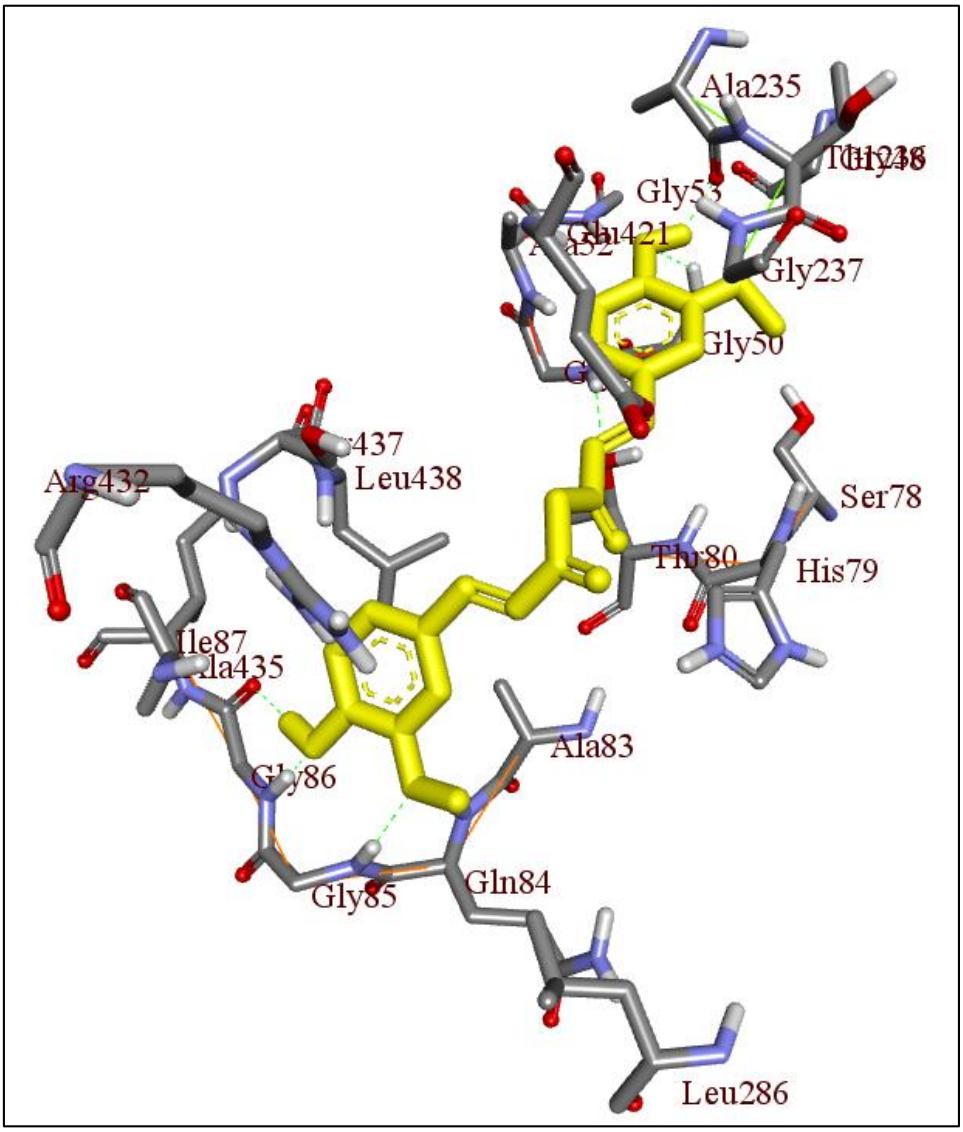

Fig 2: Interaction of Curcumin with Active binding site of Fumarate Reductase enzyme

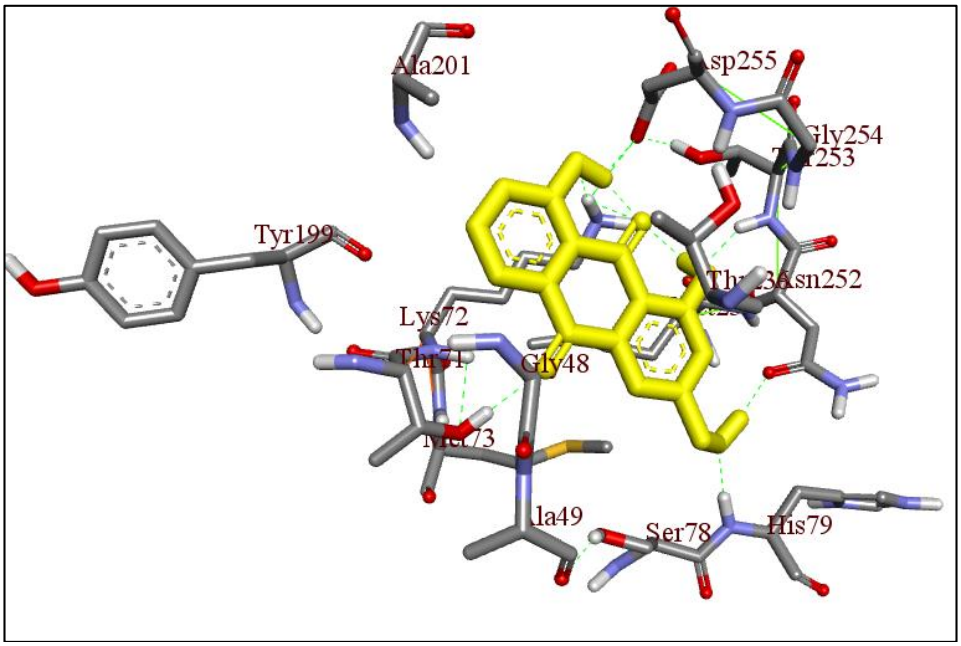

Fig 3: Interaction of Aloe emodin with Active binding site of Fumarate Reductase enzyme

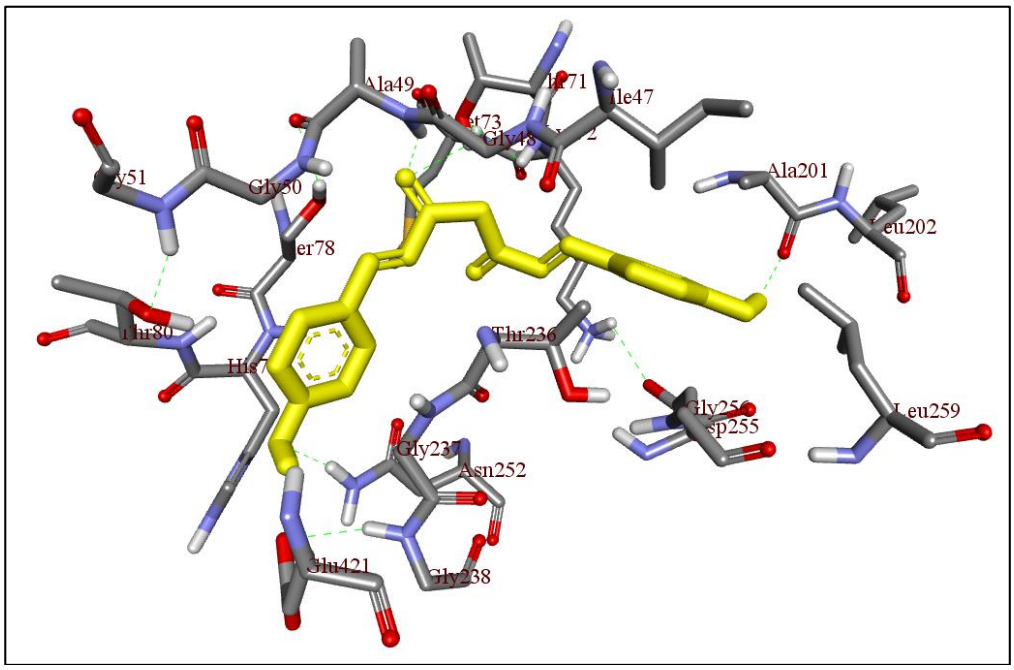

Fig 4: Interaction of Bis demethoxy curcumin with Active binding site of Fumarate Reductase enzyme

Discussion

Helminthic Infection is generally most prevalent among rural communities in warm, humid equatorial regions and where sanitation facilities are inadequate causing morbidity and mortality: it compromises nutritional status, affects cognitive processes, induces tissue reactions and causes intestinal obstruction or rectal prolapse (Lucy and Robert, 2015) [56]. The development of helminthic resistance to various groups of anthelmintics is a major problem. Compared with development of antibiotic resistance in bacteria, resistance to anthelmintics in nematodes has been slower to develop under field conditions. However, resistance is becoming widespread, because relatively few chemically dissimilar groups of anthelmintics have been introduced over the past several decades. Most of the commonly used anthelmintics belong to one of three chemical classes, benzimidazoles, imidazothiazoles, and macrocyclic lactones, within which all individual compounds act in a similar fashion. Thus, resistance to one particular compound may be accompanied by resistance to other members of the group (Jozef & Edwin, 2020) [57]. The environmental change in terms of global climate warming has further aggravated the situation due to the emergence and reemergence of helminth diseases in sheep. As a consequence the effective management of helminth diseases has become an alarming problem in sheep industry. The option of herbal anthelmintics (HA) has provided an important and viable alternative to control and treat these helminthic infections. Further, the herbal anthelmintics can be explored in reversing the AR against some of the conventional drugs in the market (Waller *et al*., 2001 and Khurshid, 2018) [58,59].

The present study was carried out to disclose the anthelminthic potential of novel herbal formulation containing rhizome of curcuma longa and leaves of cassia alata. The major phytoconstituents were screened includes Aloe emodin, kaemferol, curcumin, demethoxy curcumin and bisdemethoxy curcumin against fumarate reductase enzyme. A total of five ligands were screened for their binding against fumarate reductase enzyme, the potential of the binding between ligands and enzymes were expressed in terms of binding energy. Binding energy is released when a drug molecule associates with a target, leading to a lowering of the overall energy of the complex. The release in binding energy also compensates for any transformation of the ligand from its energy minimum to its bound conformation with the protein. Thus, lower the binding energy more stable is the complex. eg: ligand having -5.25 Kcal/mol binding energy, is more stable than ligand having 4.25 Kcal/mol binding energy. For simple understanding, Binding energy is a measure of the affinity of ligand-protein complex, or is the difference between the energy of complex and the sum of energies of each molecule separately. Intermolecular energy is the energy between non-bounded atoms that is the energy between atoms separated by 3-4 bonds or between atoms in different molecules. Torsion energy is related to dihedral term of internal energy. Inhibition constant is an indication of how potent an inhibitor is, it is concentration required to produce half maximum inhibition. Top docking pose, consider the binding energy (lowest), Inhibition constant (lowest) and more number of H-bond interactions with active site of the protein.

The binding energy of the phytoconstituents were depicted in the table and are compared with flutolanil, a standard drug. As compared to standard majority of the phytoconstituents showed lowest binding energy indicating highest inhibiting capacity for the enzyme. Among the phytoconstituents, curcumin emerged as most potent inhibitor of the enzyme producing a powerful anthelmintic activity.

Conclusion

Approximately 80% of the world's population still relies upon plants for primary health care; even today in Western medicine and despite progress in synthetic chemistry, approximately 25% of prescription medicines are still derived either directly or indirectly from plants. In the present study, an attempt was made to disclose the *In silico* anthelminthic property of a novel herbal formulation consists of curcuma longa and cassia alata respectively. The major phytoconstituents of the formulation viz.. curcumin, demethoxy curcumin, bis demethoxy curcumin, aloe emodin and kaempferol were screened for anthelminthic activity against fumarate reductase enzyme using Autodock 4.2.6.

Among the phytoconstituents screened curcumin emerged with best binding energy followed by aloe emodin and bis demethoxy curcumin. Further bioactivity guided fractionation and isolation of phytoconstituents are required to claim the underlying pharmacological activity of the herbal formulation.

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