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Research article

In silico to *In vivo* development of a polyherbal against *Haemonchus contortus*

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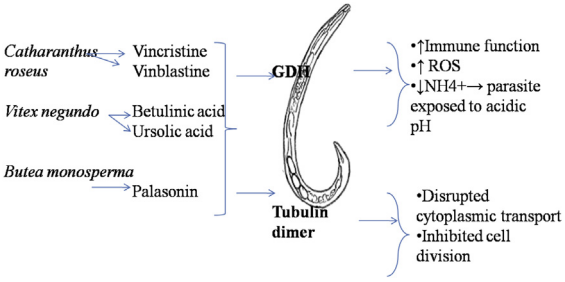
HIGHLIGHTS

- Highest binding affinity was revealed for GDH by vinblastine, ursolic acid, vincristine and palasonin and for β-tubulin by ursolic acid, betulinic acid, palasonin and vincristine compared with albendazole.
- Palasonin, vincristine, vinblastine, betulinic acid and ursolic acid showed binding at a similar site in the core of the GDH hexamer with sight variations.
- Albendazole binds near colchicine site, hindering regular interactions of α (C) chain of the tubulin dimer.
- Vinblastine binds in close proximity to laulimalide/peloruside site of the tubulin dimer.
- Both prototypes were quite efficacious in clearing the infection and keeping it at a minimum for more than 5 months.

GRAPHICAL ABSTRACT



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ABSTRACT

Haemonchus contortus is a major constraint in the development of small ruminant subsector due to significant production losses incurred by it. The present study explores the antiparasitic potential of three anthelmintic plants (*Butea monosperma, Vitex negundo* and *Catharanthus roseus (L.) G.Don*) against *H. contortus* taking albendazole as the standard.

In silico molecular docking and pharmacokinetic prediction studies were conducted with known bioactive molecules of these plants (palasonin, vinblastine, vincristine, betulinic acid and ursolic acid) against Glutamate Dehydrogenase (GDH) and tubulin molecules of the parasite. Methanolic extracts of these herbs were fractionated (hexane, ethyl acetate, chloroform and methanol) and used in *in vitro* larvicidal studies. Based on the *in vitro* data, two herbal prototypes were developed and clinically tested.

All the 5 ligand molecules showed better binding affnity for GDH and tubulin protein as compared with albendazole and shared similar binding site in the core of the GDH hexamer with slight variations. Albendazole approximately stacked against GLY190A residue, showing hydrophobic interactions with PRO157A and a Pi-cation electrostatic interaction with ARG390 along with four hydrogen bonds. Vincristine formed 2 pi-anionic electrostatic bonds with ASP158 of B and C subunits alongwith hydrogen bonding and hydrophobic interaction and an additional pi-anion electrostatic interaction at ASP158A for vinblastine. Albendazole bound to α-tubulin next to colchicine site whereas vinblastine is bound at the nearby laulimalide/peloruside site of the dimer. Betulinic acid showed lateral interaction between the H2–H3 loop of one alpha subunit and H10 of the adjacent

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alpha subunit of two tubulin dimers. Ursolic acid and palasonin bound at the intradimer N site of microtubulin involving the H1–H7 and H1–H2 zone, respectively.

The *in vitro* studies demonstrated good dose dependent anthelmintic potential. Both the prototypes were quite efficacious in clearing the infection, keeping it to a minimal for more than 5 months, probably, through direct anthelmintic effect through GDH, tubulin depolymerization and uncoupling as well as indirectly through immunomodulation along with antioxidant and anti-inflammatory properties.

1. Introduction

Haemonchus contortus is a gastrointestinal nematode which causes serious economic losses due to reduced productivity in small ruminants. It accounts for approximately 80 per cent of the parasitic load in animals [1] and has high pathogenicity [2]. Haemonchosis is mainly controlled by repetitive chemical drug administration which has led to development of resistance in the parasite and drug residues in the small ruminants, which are mainly reared as food producing animals throughout the world. Therefore, haemonchosis is considered a major constraint for the overall development of small ruminant subsector owing to production losses attributable to high mortality, morbidity and cost of treatment apart from drug residual issues.

The entire lifecycle of *H. contortus* occurs at two different places i.e., outside the host on pasture as free-living stages L1 to L3, and inside host as the L4 larvae and adult stages. Each of these stages has different requirements, regarding motility, metabolism, and the endocrine regulation. For effective host treatment, we need to target the enzymes and receptor molecules of L4 and adult parasite whose inactivation, by one or more drugs, will selectively kill parasites without harming the host animal. After entering the host animal, L3 larvae moults to the parasitic L4 and adult stages resulting in a massive surge in the expression of differential genes of structural proteins. Concomitantly, there is upregulation of genes encoding the succinate dehydrogenase subunit B and the glutamate dehydrogenase (GDH) genes through the respiratory electron transport chain [3], plausibly to maintain the redox equilibrium arising from anaerobic metabolism [4]. In addition, the parasitic GDH is also considered to be involved in immune evasion [5].

The current global trend for ecologically clean food has raised concerns over the present managemental strategies making the introduction and development of alternative remedies indispensable. Over the past few decades, the scientific community has been focusing on the evaluation of native biomolecules as immunomodulatory and prophylactic agents [6, 7]. In this sequence, anthelmintics derived from plants can be a viable and economical alternative for the treatment of haemonchus infections [8]. The numerous bioactive compounds present in herbal anthelmintics may translate into multiple mechanisms in killing the parasites, thus limiting the likelihood of developing anthelmintic resistance [9, 10]. Furthermore, they are also least likely to bioaccumulate in the host tissues and the environment [11].

Natural products are optimized bioactive molecules and represent a remarkable pharmacological diversity [12]. In fact, more than half of the drugs approved in the last 3 decades have a direct or indirect natural origin [13]. Several Indian plants have been mentioned in ancient literature for anthelmintic use [10]. Seeds of *Butea monosperma* (Lam.) Kuntze ("Palash" in hindi) contain a cantharidin palasonin, which has shown anthelmintic properties, particularly for roundworms and tapeworms [14, 15, 16]. Crude powder of the seeds at an oral dose of 1–3 g/kg to naturally infected sheep exhibited a dose and time dependent anthelmintic effect against mixed species of gastrointestinal nematodes with a maximum reduction of 78.4% in eggs per gram (epg) of feces at the highest dose in comparison to 99.1% reduction by levamisole (7.5 mg/kg), a standard anthelmintic agent. The anthelmintic activities of *Butea* species has also been reported against *Ascaridia galli*, *Ascaris lumbricoides*, earthworms, *Toxocara canis*, oxyurids, *Dipylidium caninum* and *Taenia* sp [15]. Another Indian plant, *Vitex negundo* Linn ("Lagundi" in hindi), a member of Verbenaceae family, has been prescribed as a vermifuge in the Charaka Samhita [17], and has high medicinal value with its essential oils [18], β-sitosterol [19], flavonoids [20] and triterpenoids [21]. It contains pentacyclic triterpenoids such as-ursolic acid and betulinic acid, which have shown anti-inflammatory, antitumor, antimicrobial, antibacterial and antifungal activities [22, 23].

Catharanthus roseus is used as a vermifuge by traditional healers in Madagascar [24], and has significant *in vitro* activity against free-living larvae of *H. contortus*. The leaves and the stem of the plant contain dimeric alkaloids, vinacristine and vinblastine. The plant also contains a wide range of phenolic compounds, including C6C1 compounds such as 2,3-dihydroxybenzoic acid, as well as phenylpropanoids such as flavonoids, cinnamic acid derivatives and anthocyanins [25] responsible for antioxidant activity.

Albendazole is the most frequently used anthelmintic in small ruminants. It is active against both larval and adult stages of parasitic worms. It binds to the colchicine-sensitive site of tubulin (tubulin beta-2 chain) and blocks the polymerization of tubulin proteins into microtubule, hindering the uptake of glucose leading to the depletion of glycogen stores. As a consequence, adenosine triphospate (ATP) production declines, which causes immobilization of worm followed by death (Albendazole DRUGBANK, 2018). Beta-tubulin protein of nematodes is a promising drug target for *H. contortus*. Considering the advances made in ethnopharmacological research in recent years, this study aimed to titrate the antiparasitic potential of three anthelmintic plants (*Butea monosperma, Vitex negundo* and *Catharanthus roseus (L.) G. Don*) in an optimal combination against *H. contortus* taking albendazole as a standard drug.

2. Materials and methods

2.1. Collection and extraction of plant materials

Leaves of *V. negundo* (VN) and *C. roseus (L.) G. Don* (CR) and seeds of *B. monosperma* (BM) were collected locally and identified by a botanist (Dr. Sanjay Kumar Katariya, Incharge, Department of Botany, BSA College, Mathura). The collected plant material was washed, shade dried and ground coarsely. Methanolic extraction was done by mixing about 500 gm of the coarsely ground plant material (in a porous cellulose thimble) with 2500 ml methanol at a temperature of 60 ± 5 °C using a soxhlet extractor (ASGI, India) for 20–22 cycles. The extracts (VN, CR and BM) were dried in a rotary vacuum evaporator (Heidolph).

The methanolic extracts (VN, CR and BM) were subjected to further fractional extraction using the Kupchan method of partitioning. Four fractions were collected-hexane, ethyl acetate, chloroform and methanol. The fractions thus collected were dried in a rotary vacuum evaporator (Heidolph). The extracts were stored at 0 °C to avoid loss of any volatile principles or/and their bioactivities for use in further studies.

2.2. Phytochemistry of extracts

The dried unfractionated extract was reconstituted in methanol to prepare a 1% solution and was used for phytochemical studies.

(i) *Anthraquinone*: One ml extract was heated in 1 ml of 10% ferric chloride and 1 ml of conc. HCL. Mixture was cooled and filtered with equal volume of diethyl ether and extracted with strong ammonia to give a pink colour in positive reaction.

(ii) *Alkaloid*: A drop of extract was placed on filter paper and sprayed with Dragendroff's reagent. Development of orange precipitate on the paper was indicative of presence of alkaloids.
(iii) *Glycoside*: Five mg methanolic extract was dissolved in 2ml chloroform and sulphuric acid was added to form a layer. Appearance of purple to violet colour ring at the junction indicates presence of glycosides.
(iv) *Flavonoids*: The extract was treated with few drops of dilute NaOH to give an intense yellow colour, which disappeared on addition of diluted HCl confirmed the presence of flavonoids.
(v) *Phenol*: Few drops of ferric chloride (5%) solution were added to 1 ml extract. Development of blackish-green colour indicated the presence of phenol.
(vi) *Reducing sugar*: Five ml of Fehling solution (mixture of Fehling solution I and II) was added to the extract (2 ml) and boiled (for 2 min). Development of brick-red colour indicated presence of reducing sugars.
(vii) *Saponins*: About 2–3 ml of the extract was mixed with 10 ml distilled water and shaken rigorously for about 5 min and then allowed to stand for half an hour. Presence of froth indicated positive results.
(viii) *Tannins*: few drops of 10% ferric chloride were added to 0.5 ml of the extract. Appearance of blackish blue-green colour confirmed the presence of tannins.
(ix) *Terpenoid*: The extract was dissolved in 1ml chloroform, 2–3 drops of acetic anhydride followed by few drops of conc. sulphuric acid from the side wall of the test tube and checked for the development of blue to blood red colour.
(x) *Volatile oil*: 2 ml extract was treated with 0.1 ml dilute sodium hydroxide and small volume of diluted HCl and then shaken. Formation of white precipitate was taken as a positive result.
(xi) *Protein*: 1% sodium hydroxide solution was added to 3 ml of crude extract solution in a test tube to with 1% copper sulphate solution (1–2 drops) was added. Development of violet or pink color indicated positive reaction.
(xii) *Nitrate and Nitrite:* About 0.2 ml of extract solution in a test tube was mixed with 0.8 ml of 5% salicylic acid (in con. Sulphuric acid). After 20 min, sufficient volume of NaOH solution was added to increase the pH of the solution above 12 and then cooling was allowed. Development of yellow color indicated the presence of nitrate in the extract.

2.3. In silico studies

The 3D structures of five bioactive molecules present in leaves of *Vitex negundo* and *Catharanthus roseus (L.) G.Don* and seeds of *Butea monosperma* i.e., betulinic acid, ursolic acid, vincristine, vinblastine and palasonin were retrieved from PUBCHEM (https://pubchem.ncbi.nlm.nih.gov/). The phylogenetic position of *H. contortus* in respect to the free-living nematode *Caenorhabditis elegans* was considered to select the structures of target protein and crystallography protein structure of β-tubulin (PDB ID: 6E88), and GDH (PDB ID: 1HRD) of *C. elegans* were downloaded from the PDB database (www.rcsb.org). The PDBQT structures were prepared using the computational MGL tool and molecular docking studies were carried out by using the Autodock Vina 1.5.7 [26]. The docked structures were analyzed using the visualization tool BIOVIA Discovery studio 2020 client [27].

2.4. Drug likeness calculations

The important pharmacokinetic properties and ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of the compounds were screened using the online tool DruLiTo 1.0 software to determine whether the selected phytochemicals fulfill the drug likeness conditions [28]. The sdf format of each of the phytochemicals was uploaded for the analysis.

2.5. In vitro test

2.5.1. Larvae motility test

Fecal samples from *H. contortus* infected goats reared in the ICAR-CIRG Livestock Unit, Mathura, India were collected perrectally. The infection load (eggs per gram faeces, EPG) was quantified using modified Mac-master counting technique. All experimental procedures involving the use of animals were previously approved by the Institute Animal Ethics Committee of ICAR-CIRG (working under CPCSEA) by the letter no. IAEC/CIRG/2016/4).

To collect larvae, about 20 g of feces (containing approximately 2000 EPG haemonchus eggs) were homogenized with sterile wood shavings in a proportion of 1:2 (v/v) and incubated for 7 days at room temperature. The material was moistened daily by spraying distilled water. After 7 days, L3 were recovered by their spontaneous migration using warm water (37 °C) and their concentration was corrected so that each ml volume contains about 400 larvae.

The larval motility test was conducted in different phases; the combinations of extracts and their concentrations to be used for the successive phases were decided on the basis of previous phase results. To start the study, four dilutions (25, 12.5, 6.25 and 3.125 mg/ml) for each plant extract (methanolic, chloroform, ethyl acetate and hexane fraction) were prepared in methanol. The test was conducted on a 24-well plate. In each well, 0.2 ml of desired concentration of extract was added with 1.8 ml water and 20 larvae (0.05ml). The plate was incubated at 24 °C and examined at each hour interval for 2 h.

2.5.2. Brine shrimp lethality assay (BSLT)

BSLT was done to assess the preliminary cytotoxicity of plant extracts based on the ability to kill a laboratory cultured larvae (nauplii). About 5 g of brine shrimp eggs were loaded on top of normal saline (1L). A light bulb (60–100 W) was placed a few inches away from the jar. After 20–24 h, the nauplii hatched and they were separated after the next 24 h. A suspension with a concentration of 400 nauplii per ml water was prepared.

For toxicity testing, 24-well plates were used. The test was conducted in different phases; the combinations of extracts and their concentrations to be used for the successive phase were decided on the basis of previous results. Initially, to each well, 100μl of 1% solution of each extract (single/combination) with 2 ml water and 50 μl nauplii suspension was added. The nauplii were exposed to different probable synergistic concentrations of the three plant extracts for 24 h. The number of motile nauplii was calculated for the effectiveness of the extract. The mortality endpoint of these bioassay is defined as the absence of controlled forward motion during 30 s of observation. The percentage lethality of the nauplii/larvae for each concentration and control was calculated.

$$\%\text{Death} = (\text{Number of dead nauplii/larvae})/(\text{Number of dead nauplii/larvae} + \text{Number of live nauplii/larvae}) \times 100$$

2.6. In vivo studies

The study was conducted in adult *Haemonchus*-infected goats under field conditions. The experimental protocol was approved by IAEC of ICAR-CIRG by the letter no. **IAEC/CIRG/2016/4**. Based on the *in vitro* trial, two polyherbal combinations (VN:CR:BM I-36:20:44 and II-67:99:0) were prepared using the methanolic extracts and subjected to clinical efficacy trial in field. The *Haemonchus*-infected goats (n = 6) were drenched the combinations as a total dose of 5 ml (as 1% aqueous solution w/v) on two consecutive days and the fecal egg count was monitored on regular basis up to 5 months. The number of fecal eggs were first counted using a modified McMaster technology [29] with each egg counted representing 200 eggs/g of faeces. Albendazole and potassium permanganate were given as positive and negative control, respectively.

3. Results

3.1. Phytochemistry

Qualitative phytochemical analysis of the crude methanolic extract of all the three plants is presented in Table 1.

3.2. Protein-ligand docking studies

The molecular docking of all the five ligand molecules (betulinic acid, ursolic acid, vincristine, vinblastine and palasonin) with β-tubulin and GDH exhibited significant binding energy. The amino acids involved in the protein-ligand interaction along with the energy scores are detailed in Table 2 and depicted in Figure 1. The highest binding affinity was observed for GDH by vinblastine, ursolic acid, vincristine and palasonin and for β-tubulin by ursolic acid, betulinic acid, palasonin and vincristine.

3.3. Drug likeliness studies

The different drug likeness properties such as molecular weight, number of hydrogen bonds, number of hydrogen bond acceptor and donor, hydrophilicity, topographical polar surface area prediction, molecular refractivity, number of rotatable bonds, number of atom, number of acidic groups, rotatable bond count, number of rigid bond, number of atom ring, structure alerts are explained in Table 3.

3.4. In vitro studies

At any point of the gastrointestinal tract, about 2.5–3.0 h are required for 50% emptying of the luminal contents (http://www.vivo.colostate.edu/hbooks/pathphys/digestion/basics/transit.html). Therefore, the maximum time allowed for the phytoconstituents to show their pharmacological activity in any particular segment of the gastrointestinal tract was taken as 2 h and hence, the observations were made up to 2 h post treatment. The first trial was aimed at judging the comparative larvicidal activity of the different extracts of the three selected anthelmintic plant materials (Tables 4, 5, and 6) and the BSLT results are placed in Table 7 to assess the cytotoxicity.

Two extracts each for a given plant, whose lowest concentrations (i.e. 3.25 mg/ml) showed highest mortality amongst the fractions, were selected for the next phase. This included hexane and ethyl acetate extracts of CR, chloroform and methanolic extracts of VN and chloroform and methanolic extract of BM. The higher concentration of each extract produced paralytic effect more quickly with shorter time taken for death. From the above results, it was observed that the chloroform and ethanolic extract was superior to the ethyl acetate extract although all the three extracts were endowed with anthelmintic properties. The anthelmintic activity also revealed the concentration dependent nature of different extracts.

Synergism was studied at different dose combinations of the three plant extracts at different dose levels and good mortality rate in the range of 80–92.5% was recorded and finally, two prototypes, I and II, were selected for the field trial.

3.5. In vivo studies

Two doses of 5 ml of each prototype (as 1% solution w/v) were drenched to *Haemonchus* infected goats (n = 6) on two consecutive days and the fecal egg count was monitored on a regular basis for 5 months. Albendazole and potassium permanganate were given as positive and negative control, respectively. Both the prototypes were quite efficacious in clearing the infection and keeping it minimum for more than 5 months (Table 8, Figure 2).

4. Discussion

Traditional phytomedicines represent multitarget therapy, with their different bioactive compounds working together synergistically [30, 31]. In general, phytosaponins affect the cell membrane permeability and cause vacuolization accompanied by disintegration of teguments of the parasite [32]. Alkaloids may cause paralysis of worm through the CNS effect. Alkaloids are also capable of reducing nitrate generation, and thus, can interfere in local homeostasis which is crucial for the development of helminths [33]. Phenolic compounds such as triterpenoids and tannins can hamper the energy production by uncoupling oxidative phosphorylation and interfere with the glycoprotein on the cell surface of the parasite [34]. Tannins such as palasonin may also bind to the free protein of the alimentary tract of the host or glycoprotein on the cuticle of the parasite [35]. Tannin-rich herbs also exhibit direct antiparasitic activity against internal nematodes in ruminants through augmented host resistance [36, 37], which has been clinically proven by the significant reductions of parasitic load in the herb-treated groups at necropsy. In addition, flavonoid-rich plant extracts can mitigate diseases associated with oxidative stress by their antioxidant properties [30, 38, 39] and synergistically augment the activity of condensed tannins [40] as well. Betulinic acid and ursolic acid, which are highly abundant diterpenes from *V. negundo*, have well proven antioxidant activity [41] with beneficial health-promoting effects.

H. contortus secretes a variety of proteins for survival, which are anticoagulant [42], as well as immunomodulatory [43, 44, 45, 46, 47]; and also play a role in the regulation of gut pH [48]. Amongst these proteins, a 66 kDa GDH enzyme [49] catalyzes the oxidative deamination of glutamate with the formation of α-ketoglutarate and NH_4^+. In general, helminth GDH is more active with NAD+/NADH compared with NADP+/NADPH [50] and is expressed exclusively during the blood-feeding stages of *H. contortus*. GDH has already been accepted as a potential target for anthelmintics [51] because of "significant differences" in the amino acid sequence between the host and the parasite enzyme [52]. It is also capable of inhibiting monocyte functions leading to impaired immune function [49]. Antibodies against GDH are present in the sera of *H. contortus*-infected animals, showing that the protein is well recognized by the host immune effectors cells [53]. Recently, different extra mitochondrial isoforms of GDH have been reported in organelles other than mitochondria such as rough endoplasmic reticulum, nucleus and lysosomes and are supposed to perform moonlighting functions such as an ATP-dependent tubulin-binding protein and serine proteases [54, 55]. Another plausible function may be to neutralize the reactive oxygen species released by the immune cells of the host. In

Table 1. Phytochemical constituents in methanolic crude extracts of selected plant materials.

plants	*Anthraquinone*	*Alkoaloid*	*Glucoside*	*Flavonoids*	*Phenol*	*Tannin*	*Reducingsugar*	*Saponins*	*Protein*	*Terpenoid*	*Nitricand*	*Volatileoil*
VR	++++	++	-	++++	-	++	+++	+	-	-	++	+
VN	+	+	-	++++	++	+++	++	++	-	+++	+	_
BM	+++	-	-	-	-	++	-	+	-	-	-	+

Table 2. Docking scores of different phytochemicals for *H. contortus* proteins.

Serial No.	Plant	Ligand	Affinity/Free energy (Kcal/mol)		Hydrogen bonds	
		Antibiotics/ Phytochemicals	B-tubulin	GDH	B-tubulin	GDH
1	-	Albendazole	-5.9	-6.7	ASP161D, ARG103C	ARG390A: ARG390B: SER388C: GLY190C: SER388C: ASN189C
2	*Canthranthus* roseus	Vincristine	-7.5	-8.3	THR78L, ILE76L, GLY79L, THR223L, ASN18L, GLU22L, ASN226L, ARG227L, ARG320N, ILE356N, PRO243N, GLY244N, ASP355N, GLN15L, TYR81L, THR80L	TYR188A, TYR188C, ARG390A, SER388C, SER388B, ASP158C, ASP158A, TYR188B, TYR188C
3		Vinblastine	-7.3	-8.8	GLU328D, ARG324D, GLN329D, GLY126N, ALA287D, ASP128N, GLU2N, CYS127N, ASP48N, ASN52N, GLU53N, THR285D, VAL286D	TYR188A, ARG390A, ARG390B, SER388A, SER388B, SER388C, ASP158C, ASP158A,
4	*Vitex* negundo	Betulinic acid	-7.9	-7.6	ASN214M, ARG121C, GLU88C, LEU284M, ARG119C, SER287M, ASP288M, GLU282M, ARG122C, SER292M, GLU295M, ASN291M, LYS336M	TYR188A, ASP158A, SER388C, SER388B
5		Ursolic acid	-8.0	-8.3	THR223L, TYR222L, GLY79L, GLU22L, ALA19L, TYR222L, ASN18L, GLN15L, GLU75L, THR78L	TYR188B, TYR188C, GLY190C, ARG390C, SER388C, SER388B,
6	*Butea monosperma*	Palasonin	-7.8	-8.0	TYR81L, THR80L, GLN31L, PRO37L, SER40L, GLY29L, LEU26L, CYS25L, GLU22L, ILE30L, PRO362L, PRO32L	ARG390C, TYR188B, TYR188C, SER388A, SER388C, ASP158A

Hymenolepis diminuta, GDH maintains the redox balance that gets altered as a result of anaerobic tissue metabolism [56] and might be sharing an analogous function in *H. contortus* that too exist under similar anaerobic conditions. In addition to this, the byeproduct of GDH, NH_4^+, is beneficial to the parasite against the acidic pH of the stomach [57]. So, GDH inhibition may channelize NH_4^+ production too.

GDH has two domains-N and C, that are separated by an active site cleft, which also contains the substrate binding site at its depth [58]. Domain C, with residues 4–181 and 400–421, is responsible for the assembling of the enzyme hexamer while the *N domain* (182–399 residues) is the glutamate binding domain [59]. Of these, 193–204 and 383–393 residues are essential for glutamate binding [60]. The N and C domains bear a NAD + binding domain on their top with its 48 residue antenna. During the catalytic cycle, domain C rotates clockwise closer to domain N and thus rotates the NAD + domain down on the substrate and coenzyme with a conformational change in its antenna as the cleft opens and closes [61]. To qualify as a drug, a molecule should possess affinity for the target molecule i.e. certain vital pharmacophore features such as hydrogen bond accepting ability (e.g., hydroxyl or carbonyl groups) or donor groups (e.g., hydroxyl or amide groups), charged groups (e.g., carboxylates), and hydrophobic aromatic groups (e.g., cyclohexyl or phenyl ring or other aromatic ring systems) with which it can interact with its proposed receptor protein. Surprisingly, all the 5 ligand molecules (palasonin, vincristine, vinblastine, betulinic acid and ursolic acid) showed binding at the similar site in the core of the hexamer but with slight variations. Albendazole approximately stacks against GLY190 residue of the A subunit, making hydrophobic interactions with PRO157A and a Pi-cation electrostatic interaction with ARG390 along with four hydrogen bonds. Vincristine forms 2 pi-anionic electrostatic bonds with ASP158 residues of B and C subunits along with hydrogen and hydrophobic interaction. Still better binding affinity was displayed by vinblastine, which may be attributed to an additional pi-anion electrostatic interaction at ASP158A. The absence of hydrophobic and electrostatic interactions in betulinic acid might be plausibly the reason for its low binding affinity compared with that of ursolic acid and palasonin.

Microtubules in helminths are involved in cell division, cell motility and cytoplasmic transport and are a lucrative target for benzimidazoles. It is probably the disruption of cytoplasmic transport that has the greatest anthelmintic effect. These microtubules are heteropolymers containing an equal ratio of alternating α (A, C, H, I, L, and M chains) and β (B, D, J, K, N, and O chains) tubulin protein, in a helical arrangement along with guanine nucleotides. The heterodimers exist as head-to-tail arrays of bent tubulin conformation in microtubule depolymerization peels [62], where longitudinal contacts are maintained after lateral contacts are broken. The lateral contacts are mediated by a series of loop-loop interactions [63, 64], and are the most labile of the microtubule lattice interactions. The biological functions of microtubules are mainly regulated by polymerization and depolymerization of the α- and β-tubulin heterodimers [65] which in turn is regulated by GTP hydrolysis. The microtubule polymerization can be suppressed by binding of the tubulin either to a depolymerizer [66, 67, 68] and/or to destabilizing drugs [69, 70], which are probably biased towards a bent conformation.

Within the tubulin heterodimer, the N-terminal domain (residues 1–205) forms a Rossmann fold, with α -helices H1 and H2 are on one side, and helices H3, H4 and H5 on the other. The strand B6 (residues 206–381) leads to the intermediate domain of the heterodimer, with a mixed β-sheet and five surrounding helices. The B7 β-strand interacts with the β-sheet in the N-terminal domain while the loop connecting B7 and H9 preserves strong lateral contacts in α-tubulin. The C-terminal domain, formed by helices H11 and H12, overlay the previous domains, and forms the surface of the molecule. During polymerization, the α-tubulin subunit of the incoming dimer hydrolyses the exchangeable GTP of the terminal β-tubulin subunit [71], which ultimately leads to a compaction around the GTP-site nucleotide (E-site) at longitudinal interfaces, resulting in an energetically unfavorable longitudinal translation of the α-tubulin intermediate domain ultimately, moving it as a unit with the C-terminal base of H7 and getting effectively tucked up into the dimer and most significantly, leading to rotation of the intermediate domain and a translation of H7 [67,70,71]. The internal strain within the tubulin dimer results in a rearrangement in the intermediate domain [72], and helices H11 and H12 in both α- and β-tubulin subunits move away from the lumen. The αH12 helix constitutes a part of the binding site for the kinesin and cytoplasmic dynein motors [73, 74, 75]. The resultant displacement of the C-terminal helices in both α– and β–tubulin subunits is indicative of the potential of depolymerizing ligands of this region as anthelmintic agents. Microtubule stabilizing agents, taxol or peloruside -like compounds bind to the lateral side of tubulin [68] while

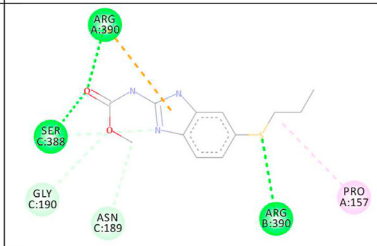
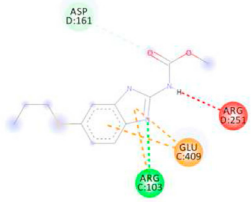
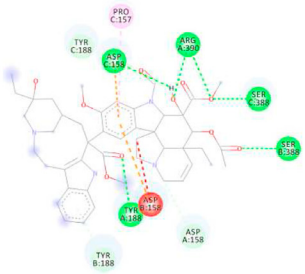
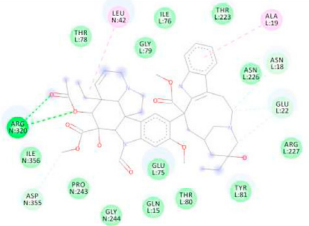
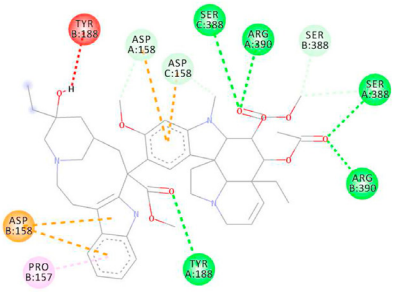
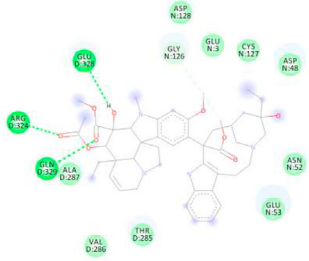
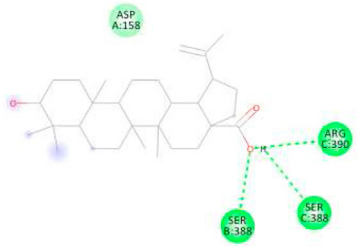
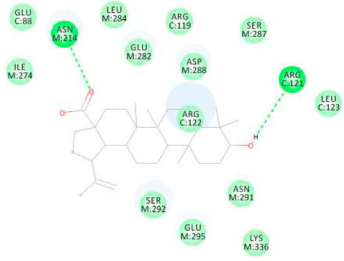
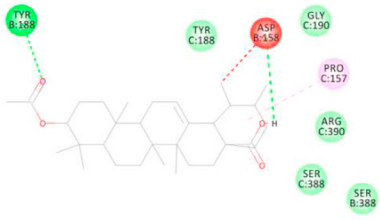
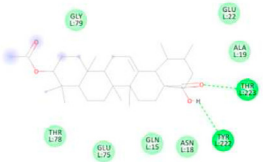
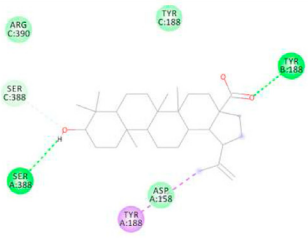
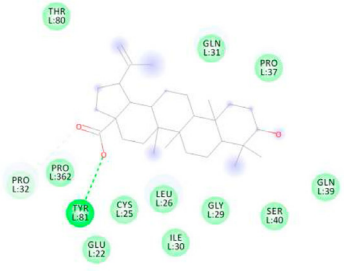
Ligand/receptor protein	GDH	Tubulin
Albendazole		
Vincristine		
Vinblastine		
Betulinic acid		
Ursolic acid		
Palasonin		

Figure 1. Docking of haemonchus proteins with different phytochemicals.

Table 3. Pharmacokinetic profile of different phytochemicals as calculated using DruLiTo software.

Phytochemicals	MW	logp	Alogp	HBA	HBD	TPSA	AMR	nRB	nAtom	nAcidicGroup	RC	nRigidB	SAlerts	nAromRing	nHB	LipinskiesRuleofFive	Ghose_filter	CMC − 50LikeRule	VeberRule	MDDRLikeRule	BBBLikenessRule	UnweightedQED	WeightedQED
palasonin	182.06	-0.318	-0.918	4	0	52.6	37.22	0	23	0	3	15	4	0	4	✓	×	✓	✓	x	✓	x	x
betulinic	456.36	9.407	2.35	3	2	57.53	131.75	2	81	1	5	35	2	0	5	x	x	x	✓	x	x	✓	x
ursolic	498.37	9.694	1.863	4	1	63.6	141.41	3	86	1	5	37	3	0	5	x	x	x	✓	x	x	✓	x
vinblastine	810.42	3.056	-1.25	13	3	150.34	227.52	10	117	0	9	57	5	3	16	x	x	x	x	✓	x	x	x
vincristine	824.4	1.887	-2.07	14	3	167.41	227.72	11	116	0	9	57	6	3	17	x	x	x	x	✓	x	x	x

MW-molecular weight, logP- octanol–water partition coefficient, Alogp- Ghose-Crippen-Viswanadhan octanol-water partition coefficient, HBA-hydrogen bond acceptor, HBD-hydrogen bond donor, TPSA-topological polar surface area, AMR- Atom Molar Refractivity, nRB- number of Rotatable Bonds, nAtom-number of atoms, nAcidicGroup-number of acidic groups, RC- rotatable bond count, nRigidB- number of rigid bond, nAtomRing: number of Atom Ring, nHB: number of Hydrogen Bond, SAlerts:Structure alerts.
CMC Comprehensive Medicinal Chemistry, MDDR-modern drug data report, BBB – blood brain barrier, QED- Quantitative Estimate of Drug-likeness.

microtubule destabilizing agents bind to the longitudinal interface between the α and β tubulin subunits, causing conformational shifts of the consecutive α, β-tubulin heterodimers, facilitating microtubule disassembly [69, 70]. The different possible binding sites for microtubule targeting agents are recognized as the colchicine site at the α/β intra-heterodimer interface [76], the vinca domain at the α/β interdimer interface [77]), the rhizoxin/maytansine domain [78], the epothilone domain [79], the taxane domain [80], and the laulimalide/peloruside domain [81]. Benzimidazoles have been found to bind to the colchicine binding site of tubulin and inhibit the polymerization of microtubules [82]. In the present study, albendazole bound nearby colchicine site via hydrogen bonding and electrostatic attractions involving ARG103C, GLU409C and ASP161D residues, hindering the regular physiological interactions on the lateral and longitudinal surfaces of the α- (C chain) and β- (D chain) tubulin subunits, essential for polymerization in the microtubule lattice. Significant differences exist between mammalian and protozoal parasite tubulins at the colchicine-binding site, which account for the selectivity of the benzimidazoles. *Catharanthus* alkaloids have two distinct binding sites on microtubules: binding with high affinity at the heterodimer ends, and with low affinity to the lateral surface [83, 84]. They also augment the affinity amongst heterodimers, and forms spiral aggregates [83], leading to loss of physiological function. Vincristine binds near the intermediate domain and the H7 loop of alpha subunit whereas vinblastine showed binding near the laulimalide/peloruside site and appears to play an important role in the lateral arrangements of the microtubule dimers. Vinblastine was sandwiched between subunits β1 and β2 towards the α-subunit and mainly interacted with helix H3 and strand S2 of β1 subunit and helices H9 and H10 of β2 subunit. This lateral occupancy between β subunits is in accordance with the findings of previous studies [68]. Both van der Waals and hydrogen bonding interactions were observed. In contrast to this, vincristine bound in the cervix formed between H1–H2 and H7 helices of the α subunit and strands S8 and S9 of β subunit of same heterodimer, involving hydrophobic (pi-alkyl) interactions at ALA19L and LEU42N as well.

Betulinic acid also showed interactions between the lateral surfaces of the adjacent dimers-between the H2–H3 loop of one α-subunit and H10 of the adjacent α-subunit. The ursolic acid and palasonin bounded at the intradimer N site of microtubulin involving H1–H7 and H1–H2 zone, respectively like vincristine but at a slightly posterior location.

Uncoupling proteins (UCPs) are integral cell membrane proteins that dissipate the proton electrochemical gradient generated during electron transport via the mitochondrial respiratory chain, resulting in energy loss in the parasite and may aid its killing. In general, uncoupling is caused primarily by an interaction of a weakly acidic molecule with the membrane phospholipid, making the membrane permeable to H^+, resulting in uncoupling. Phenols, benzimidazoles, N-phenylanthranilates, salicylanilides, phenylhydrazones, salicylic acids, acyldithiocarbazates, coumarins, and aromatic amines, being weak acids, are known to induce uncoupling [85, 86, 87], attributable to their protonophoric actions. Both betulinic acid and ursolic acid are well known to cause compromised mitochondrial function, increased expression of mitochondrial uncoupling proteins (UCP) 1 and 2 [88,89]. In fact, betulinic acid induces a metabolic reprogramming that is highly indicative of the Warburg effect (aerobic glycolysis favored over mitochondrial respiration) in tumor cells [90].

The adoption of drug-likeness concepts early in the drug discovery process reduces attrition rates [91]. The present routine anthelmintic, benzimidazoles generally exhibit from poor solubility and poor absorption. For a molecule to be an ideal anthelmintic, it should reach the site of predilection of the parasite and once it has exerted its pharmacological effect, it should be effluxed out of the host body easily. Several *in silico* tools successfully predict the kinetic profile of the molecule inside the host on the basis of molecular weight, number of hydrogen or rotatable bonds, polarity, and flexibility [92]. No single parameter or method is fail-safe but the sum of several different molecular descriptors can effectively give an idea of the profiling of the key features for a drug [93]. *H. contortus* resides in the abomasal lumen so an orally effective drug

Table 4. Mean Per cent Larvae mortality achieved after exposure to different concentrations of plant extracts individually.

Extract	Concentration (mg/ml)	1st h post treatment				2nd h post treatment			
		hexane	Ethyl acetate	chloroform	methanol	hexane	Ethyl acetate	chloroform	methanol
VR	25	80	70	75	80	100	100	100	100
	12.5	65	70	60	75	100	100	100	100
	6.25	65	40	45	60	85	75	85	90
	3.125	45	30	20	45	65	50	40	40
VN	25	60	70	90	80	100	100	100	100
	12.5	45	20	85	65	100	100	100	100
	6.25	30	15	85	50	80	80	90	75
	3.125	10	5	40	50	50	65	80	65
BM	25	75	85	65	70	90	100	100	100
	12.5	50	75	40	60	65	75	100	100
	6.25	35	60	25	35	50	75	85	95
	3.125	20	20	5	35	30	55	65	70

Table 5. Mean Per cent Larvae mortality (in duplicate) achieved after exposure to different plant extracts in 1 % concentrations (100µl) individually and in combination.

Extract	1 h			2 h		
BM	chloroform	Chloroform + methanol	methanol	chloroform	Chloroform + methanol	methanol
	20	35	55	30	50	60
	15	40	75	25	55	85
VR	hexane	Hexane + ethyl acetate	ethyl ac	hexane	Hexane + ethyl acetate	ethyl ac
	70	40	35	75	60	60
	65	45	40	85	50	50
VN	chloroform	Chloroform + methanol	methanol	chloroform	Chloroform + methanol	methanol
	10	60	45	20	70	50
	15	55	40	30	75	55

Table 6. Per cent Larvae mortality achieved after 2 h exposure to 1% concentrations of plant extracts individually and in combination.

Extract	BM methanol (50µl)	VR hexane (50µl)	VN chlor (50µl)	VN methanol (50µl)
BM methanol (50µl)	45	70	60	55
VR hexane (50µl)	55	70	65	100
VN chlor (50µl)	45	65	60	75
VN methanol (50µl)	65	55	65	75

would be the most suitable with minimal host interactions and toxicity. Therefore, Lipinski's Rule of 5 (Ro5), associated with orally active drugs could effectively predict the likelihood of the molecule to act as an anthelmintic drug [94]. It states that molecules with >500 Da molecular weight, >5 hydrogen-bond donors, >5 log P, and >10 hydrogen bond acceptors have poor absorption. Log P (partition coefficient between octanol and water) indicates the lipophilicity of the drug candidate which is a major determinant of the drug-likeness and a value of log P > 0.8 is required to guarantee sufficient membrane permeability and renal clearance [95, 96]. An increase in lipophilicity, as observed in case of vinblastine and vincristine, might increase the pharmacodynamic potency, mostly due to the involvement of entropically favored exchanges between the hydrophobic functionalities of the drug and the recognized receptor [97]. However, a very high lipophilic nature (log P approaching 9), as shown by betulinic acid and ursolic acid, reflects a tendency for receptor promiscuity along with a less favorable pharmacokinetic profile and may eventually lead to toxicity [92]. Only palasonin qualified all the Ro5 parameters but the plausibility of other molecules being drugs cannot be completely ruled out as there are several antibiotics, antifungals, vitamins, and cardiac glycosides which are a part of successful therapy even though they violate Ro5. This is partially due to intramolecular hydrogen bonding and partially due to the fact that they are substrates for transporters. As far as the Lipinski's limit of molecular weight of 500 was concerned, antiparasitic drugs are typically larger and more complex than therapeutics from almost every other therapy area, except cancer [98]. Therefore, oral administered anti-infective drugs from natural origin often do not comply with this rule of Lipinski.

Hydrogen bonding patterns are critical to the packing of protein receptors and hence to the formation of the cavities and crevices that are the binding sites for drugs. Within a single ligand, it is very difficult to accommodate more than 2–3 hydrogen bond donor groups with the

Table 7. Synergistic studies for best effective dose combination determination on basis of mortality percentage using Brine Shrimp Lethality Assay.

	hrs post treatment	Volume	VN ethyl ac			BM ethyl ac		control
			75 µl	50 µl	25 µl	75 µl	50 µl	
VR ethyl ac	1 h	25 µl	30	22.5	10	10	7.5	0
		50 µl	22.5	10	7.5	5	2.5	0
	24 h	25 µl	85	47.5	37.5	42.5	47.5	0
		50 µl	92.5	42.5	32.5	82.5	80	0

Table 8. Post treatment egg count per gram faeces observed in field *Haemonchus* infected goats after drenching two doses of prototypeon consecutive days.

Treatment group	Days post treatment							
	0	3	5	10	30	60	100	150
albendazole	3100.00 ±588.52	500.00 ±153.27	400.00 ±149.97	850.00 ±273.80	1350.00 ±435.80	1375.00 ±259.03	1250.00 ±405.50	1225.00 ±425.50
pot permagnate	1800.00 ±437.52	1750.00 ±535.15	1300.00 ±513.70	1275.00 ±243.11	1100.00 ±432.34	1225.00 ±444.46	900.00 ±345.62	800.00 ±325.51
I	1750.00 ±870.16	1450.00 ±957.14	1450.00 ±646.78	1075.00 ±291.70	250.00 ±77.44	175.00 33.53	100.00 ±0.00	100.00 ±0.00
II	2250.00 ±552.61	1200.00 ±524.77	900.00 ±249.95	550.00 ±232.33	700.00 ±540.72	150.00 ±31.62	100.00 ±0.00	100.00 ±0.00

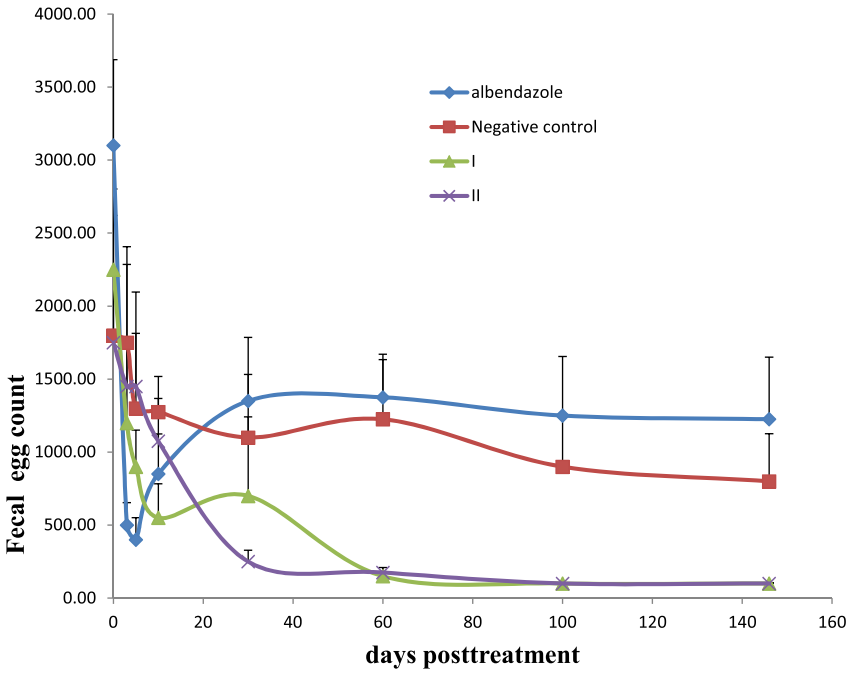


Figure 2. Total hemonchus egg count per gram of goat faeces with respect to days post treatment with CIRG-A1 and CIRG A2.

specific orientation needed for the maximum enthalpic energy benefit [99] due to entropy–enthalpy compensation [100]. Therefore, as the number of potential hydrogen bond donors in a ligand increases, they are more likely to have a negative impact on ligand-protein binding with the directionality of hydrogen bonds imposing further restrictions [101]. This observation is very consistent with the common medicinal chemistry (CMC) assessment regarding complications to improve drug strength by addition of hydrogen bond donor groups. Veber's rule [102] and Ghose's rule [103] have since been proposed, to include more properties associated with bioavailability, such as polar surface area, and to improve upon the concept proposed by Lipinski. All the molecules missed the Ghose filter by 2–3 parameters and Veber's rule [102] is generally considered to be a far too conservative measure. Palasonin, betulinic acid and ursolic acid qualified the Veber's criteria while Vinca alkaloids could find a place in the modern drug data report (MDDR). More recently, the quantitative estimate for drug-likeness (QED) has been proposed as an alternative to rule based methods [104]. The QED values can range anywhere between 0 (all properties unfavorable) and 1 (all properties favorable). Palasonin also qualified the comprehensive medicinal chemistry (CMC) database list of medicinal molecules while betulinic acid and ursolic acid qualified for the unweighted QED.

The total polar surface area (TPSA) illustrates good correlation with passive transport through cell membranes, allowing estimation of the transport properties of drugs. Indeed, the 3 molecules with TPSA values less than 140 (palasonin, betulinic acid and ursolic acid), and atom number and molar refractivity close to 70 and 130, respectively, can be lucrative drug options. The Lipinski, Ghose and Veber filters place a minimal emphasis on the basic structure groups. In addition to this, the number of rotatable bonds (RB) and aromatic character are also used as a measure for molecular flexibility [102]. According to MDDR, the probability of finding a 'druglike' compound is high when number of aromatic rings $\geq$3, a criteria fulfilled by all the five ligand molecules. The therapeutically useful herbal ligands usually appear to cluster together in nature but the clustering of ligand cavities on the receptor is not well predicted either by sequence or fold space. So, differences exist in the similarities in sequence-, fold- and cavity space [105], and as a consequence, cross reactivity is observed for ligands of proteins of unrelated sequence or fold. Besides, the microtubule targeting agents can synergize with one another like the dose-dependent synergistic interaction between ABZ and CLC [106].

Clinically combined drug therapies aim to augment the total effects in treating the disease condition, reduce the side effects via dose-sparing of the active components, or address the different metabolic interdependence, mediators or risk factors of diseases through a variation of independent biomolecular targets. Synergy research on herbal medicine is still in its infancy. *In vitro* larval motility tests have been accepted as a rational and practical means to study novel anthelmintic drugs [107, 108]. The test allows the bioactive material to interact directly with the stage of the parasitic life-cycle. Therefore, *in vitro* synergistic combinations were attempted amongst the extracts under study.

An effective anthelmintic should account for minimal 99% inhibition of larval motility [109]. Kamaraj and Rahuman, [110] reported 100% egg hatching inhibition by ethyl acetate extract of *C. roseus* [110]. All the extracts showed good larval mortality. There are reports describing the better anthelmintic potential of chloroform, ethyl acetate and ethanol extracts. In the present study, the ethyl acetate fraction gave better results for CR while the chloroform and methanolic extracts of VN and BM had higher efficacy. Similar findings have earlier been reported by Rajan and Robertson (2017) for *Raphanus sativus* leaf extract [111]. The order of activity was chloroform, ethanol extract followed by ethyl acetate extract. The concentration dependent nature of different extracts was also evident. Potency of the extracts was found to be inversely proportional to the time taken for paralysis/death of the worms. The recent abundant evidence confirmed the involvement of oxidative stress in the pathogenesis of several acute, chronic disorders and diseases. Exogenous antioxidants such as tannins, phenols, flavonoids and terpenoids input as the prototypes I and II were expected to certainly help the endogenous enzymatic and nonenzymatic antioxidant defense systems in correcting the redox imbalance and to control the production of reactive oxygen or nitrogen species. Tannins such as palasonin interfere with the neuromuscular coordination of the larvae [36, 112, 113] and cause paralysis or reduce the motility of the larval stages, which may indicate a clinical representation of lack of energy or cytoplasmic transport of mediators owing to GDH and tubulin inhibition.

The antioxidant potential of the prototypes may be attributed to the three constituent extracts, each loaded with bioactive antioxidants. BM seeds contain palasonin, butin, monospermoside, isomonospermoside, allophanic acid, α-amyrin, β-sitosterol and β-sitosterol-β-D-glucoside and nitrogenous acidic compounds. The crude powder of BM seeds had exhibited significant *in vitro* anthelmintic activity against gastrointestinal trichostrongylid nematodes in sheep [15] whereas alcoholic and ethyl acetate extracts of BM leaves exhibit activity against earth worms, roundworms and tapeworms and *C. elegans* [114]. There are also studies reporting the anthelmintic efficacy of the aqueous extracts of BM seeds against *H. contortus* of sheep and goats [115]. In addition, VN leaves contain various chemical classes such as alkaloids, tannins, flavonoids and carbohydrates alongwith betulinic acid and ursolic acid and possess good anti-oxidant potential and antifungal activities [116]; anthelminthic properties [117]; dysmenorrheal properties [118]; pain suppressing activity [119]; anti-hyperglycemic activity [120]; anti-filarial [121]; anti-bacterial [122] and tranquilizing insecticidal properties [123, 124]. CR is also a good source of non-enzymatic and enzymatic antioxidants [125]. The leaves have antibacterial and antidiabetic properties [126, 127]. It also contains a wide range phenolic compounds [25] responsible for antioxidant activity.

Although synergistic effects have been demonstrated in numerous *in vitro* pharmacological studies, laboratory findings do not always represent clinical therapeutic superiority. Therefore, the clinical benefits of multi-component combinations must be subsequently confirmed in rigorous clinical trials. Two doses of 5 ml of each prototype (as 1% solution w/v) was adminstered to *Haemonchus* infected goats (n = 6) on two consecutive days and the fecal egg count was monitored on regular basis up to 5 months. Both the prototypes were quite efficacious in clearing the infection in CIRG shed as well as field *Haemonchus* infected animals and maintained it to a minimal for more than 5 months. The ability to expel *haemonchus* infection also depends on the development of a protective acquired immune response; however, the level of immunity depends on age, nutritional status and host genotype [128]. Relatively high clinical efficacy, as compared to our *in vitro* tests, suggested that the herbal mixtures could have an indirect antiparasitic effect *in vivo* and might be able to promote the resistance of the host to parasitic infection only in the longer term [129]. Tannins, such as palasonin, have been reported to contribute to the anthelmintic property through immune response as well [39, 130] along with antioxidant and anti-inflammatory properties [38, 131].

To conclude, the antiparasitic mechanism of plant extracts usually differs with respect to the type of parasite, its stage of development, and the phytochemistry of the plant. Multiple bioactive substances present in polyherbal extracts can target multiple metabolic and physiological processes of the different stages of *H. contortus*, preventing further development and perpetuation thus representing a potential source of a new anthelmintic drug. The prototype I showed slightly superior clinical profile compared with the prototype II and perhaps, involves the immunomodulation of the host as well. Multi targeting of the parasite and the host immune mechanism as well, can certainly be of greater clinical benefit to eliminate the parasite and promote the defense mechanism of the host at the same time, giving an additional benefit to the host against other pathogens as well.

Declarations

Author contribution statement

Anu Rahal: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

D.K. Sharma: Conceived and designed the experiments; Analyzed and interpreted the data.

Ashok Kumar: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Nitika Sharma: Analyzed and interpreted the data; Wrote the paper.

Deen Dayal: Performed the experiments.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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