

## Pharmacognosy of *Ecbolium viride*: A Review

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### ABSTRACT

In India use of different parts of several medicinal plants to cure specific ailments has been in vogue from ancient times. Although the traditional system of medicine has large number of plants with various medicinal and pharmacological properties and are yet to be discovered which represents a priceless tank of new bioactive molecules. *Ecbolium viride* (Forssk).Alston belonging to the family Acanthaceae is a perennial woody under shrub. Traditionally different parts of this plant like roots, leaves, and stem as well as whole plant are used in folklore medicine for various ailments like tumors, jaundice, menorrhoea, rheumatism, inflammation. Apart from the research and pharmacological properties of *Ecbolium viride*, its synonym *Ecbolium linnaenum* is also succinctly discussed here. Some of its medicinal properties have been mentioned in Siddha, Ayurveda and Unani system of medicine. This review attempts to encompass the adequate information to develop suitable therapeutics and bioactive molecules out of these plant parts.

**Keywords:** *Ecbolium viride*, *Ecbolium linnaenum*, Acanthaceae, Phytochemistry, Pharmacology.

### INTRODUCTION

Recently many countries had focused to exploit the medicinal plants and their products as a potential candidate for the management of diverse ailments and disorders. Plants are used medicinally in different countries and are a source of many potent and powerful drugs<sup>1</sup>. Nowadays, people have accepted plants as a cheaper alternative to orthodox medicine<sup>2</sup>. One might expect plants used in traditional medicine to be safe over a long period<sup>3</sup>. Hence, an evaluation of the toxicity, Cytotoxicity and mutagenicity are important in all scientific studies<sup>4</sup>. There is an urgent need to discover novel, effective plant-based drug to the increasing problem of drug resistance. In this scenario, detailed review of *Ecbolium viride* (Forsk.) Alston. (Acanthaceae) as well as *Ecbolium linnaenum*, its synonym is well described. Traditionally different parts of the plant like roots, leaves, stem and whole plant which are used in folklore medicine for several medicinal purposes like cancer, jaundice and rheumatism<sup>5-6</sup>. It also possesses pharmacological properties such as anti-microbial, anti-diarrhoeal, hepatoprotective and antioxidant properties. Even though it is considered as an undiscovered plant, its medicinal uses and pharmacological actions are of utmost importance in human usage these days.

Folklore medicinal value of *E. viride*: In folk medicine, ethanolic (50%) extract of the plants are used to treat cardiovascular disease<sup>7-10</sup>. Decoction has been reported to possess many ritual uses such as in jaundice, menorrhoea, rheumatism<sup>11-12</sup>. Aqueous extracts of dried roots of the plant are used for menorrhagia, rheumatism and jaundice<sup>13</sup>. This plant is widely used in Indian traditional medicinal system (Siddha, Ayurveda, Unani) and Folk. Leaves are used in gout and dysuria; decoction of leaves

for stricture<sup>14-15</sup>. All parts of the plant are used for gout and dysuria<sup>16</sup>. Crude extracts of plant exhibited a significant antimicrobial activity and in the treatment of some diseases as broad-spectrum antimicrobial agents<sup>17</sup>. Leaves, roots and flowers contain glycoflavones, Orientin, Vitexin, Isoorientin, Isovitexin and other flavones<sup>18</sup>. Leaf poultice is used for Leprosy. Decoction of leaves and flowers are taken internally as diuretic and gonorrhoea. Bark root is used for pulmonary problems<sup>19</sup>. The root juice of this plant was utilized by the rural people in Tirunelveli district of Tamilnadu for the treatment of jaundice by the local native practitioner. Root juice is used as anti-helmenthic and also to treat premenstrual colic<sup>20</sup>.

Taxonomy<sup>21-23</sup>

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Scrophulariales

Family: Acanthaceae

Genus: *Ecbolium*

Species: *Ecbolium viride* (Forssk).Alston

Common name: Green Shrimp plant

Synonym: *Ecbolium linnaenum*.

Vernacular names<sup>24-25</sup>

English: Blue fox Tail Nail Dye

Telugu: Nakka toka

Tamil: Nilambari

Hindi: Udajati

Malayalam: Karinkurinji

Kannada: Kappu bobballi (kappu karni)

Sanskrit: Sahacharah

Bengali: Neel Kantha

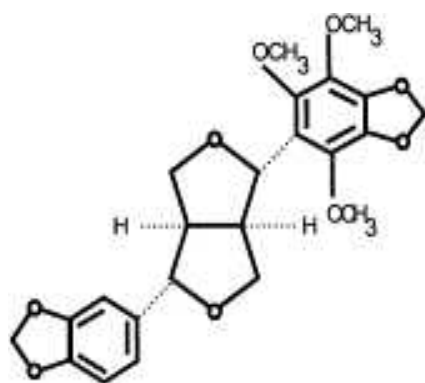


Fig.1: Ecbolin A - 6-(3,4-methylenedioxyphenyl)-2-(2,5,6-trimethoxy-3,4-methylenedioxyphenyl)-3,7-dioxabicyclo[3.3.0]octane isolated from *E.viride* roots

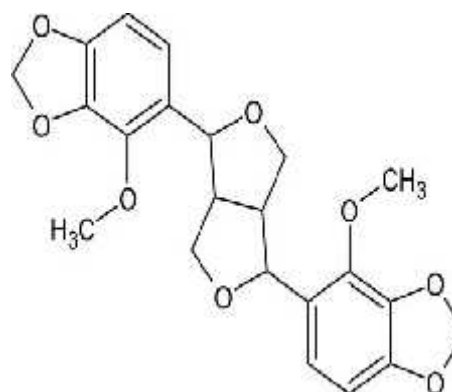


Fig.2: 4-methoxy-5-[4-(4-methoxy-1,3-benzodioxol-5-yl)perhydro-1H,3H-furo[3,4-c]-furan-1-yl]-1,3-benzodioxole.

Table 1. Preliminary Phytochemical screening of various organic solvents and aqueous extract of *E. viride*

Compounds	Leaf extracts				Stem extracts					
	Acetone	Ethanol	DCM	Ether	Aqueous	Acetone	Ethanol	DCM	Ether	Aqueous
Alkaloid	+	+	+	-	+	+	+	+	-	+
Carbohydrate	+	+	-	-	+	+	+	-	-	+
Flavonoid	+	+	+	+	+	+	-	+	+	+
Glycoside	+	+	+	+	+	+	-	+	+	+
Phenol	+	+	-	-	+	+	+	-	-	-
Phytosterol	+	+	+	-	+	+	+	-	+	-
Protein	+	+	-	-	+	+	+	+	-	+
Resin	+	+	-	-	+	+	+	-	-	-
Saponin	+	+	-	-	+	-	+	-	-	+
Tannin	+	+	-	-	+	+	+	-	-	+
Thiol	+	+	+	-	+	+	+	+	-	+

'+' Represents presence of the phytoconstituent; '-' represents absence of the phytoconstituent

Botany of *E.viride*: From botanical description *E.viride* is a perennial woody under shrub. Stems are erect glabrous, grows up to 1-3m tall with erect branches. Leaves are large (11.5-15cm), arrangement is opposite-decussate, leaf type is simple shape is elliptic-ovate to obovate, margin is entire or undulate, apex is acute, pubescent, base attenuate tapering to base. Calyx lobes are 5, unequal, decurrent, lanceolate, valvate. Flowers are large, sessile, present in opposite pairs, spikes nearly sessile, 5-25 cm long; corolla tube is 3.8 cm long, slightly dilated and laterally compressed at throat. Corolla is tubular and 2-lipped, lobes are 5. Stamens are 2, epipetalous, exserted. Ovary is bicarpellary, bilocular; ovules 2 per locule; style filiform, hairy below; stigma capitate. Capsules ovoid, compressed, narrowed at base; seeds 2-4, orbicular, tuberculate. Fruits are ovoid and capsule consists of two seeds. Stems are several, straight, jointed and swelled above the joints. They can be easily identified by their intense green leaves and greenish blue flowers<sup>26-28</sup>.

Geographical distribution of *E.viride*: The native range of *E. viride* extends from South and North eastern peninsular part of the country found occasionally in plains and forests of India. It is also found in Arabia, Malaysia, Srilanka and tropical Africa<sup>29</sup>.

Chemistry: The plant extract contains several organic compounds which are mostly heterocyclic aromatics and anti oxidants.

Four glycoflavones luteolin, orientin, vitexin and isoorientin have been isolated from the ethanolic extract of the root, flower and leaves of the plant<sup>30</sup>.

A furofuran type of unsymmetrical lignan named Ecbolin A, from the chloroform extract of the root of *E. viride* has been isolated<sup>31</sup>.

The ethyl acetate root extracts of *E. viride* contains a novel hetero furanoid compound 4-methoxy-5-[4-(4-methoxy-1,3-benzodioxol-5-yl)perhydro-1H,3H-furo[3,4-c]-furan-1-yl]-1,3-benzodioxole<sup>32</sup>.

Novel flavone glycoside [luteolin 7-O-(2'' sinapoyl) glucoside] has been isolated from the ethyl acetate extract of root<sup>33</sup>.

Ethanolic extracts of leaf when subjected to GCMS analysis few probable compounds were reported as follows<sup>34</sup>:

R)-4-(1',1'-Dimethylethyl)-1,3,2-dioxathiolane-2-one  
Neophytadiene  
3,5-Dioxohexanoic acid  
3-Chloromethylfuran  
9,12,15-Octadecatrienoic acid, methylester, (Z,Z,Z)-  
(CAS)  
1-(Cyclohexan-1-yl)but-3-ene

An active compound Ecbolin A (furofuran type of lignan) was isolated from ethyl acetate extract of roots<sup>35</sup>.

Phytochemical characters: The preliminary phytochemical screening of the plant material revealed

Table 2. Organoleptic study of *E. viride* leaf powder

Sl. No	Parameters	Characteristic feature
1	Taste	Light bitter
2	Colour	Green
3	Smell	Normal
4	Appearance	Coarse Powder

the presence of alkaloids, carbohydrates, glycosides, phenols, phytosterols, thiols, gums & mucilage, flavonoids, terpenes, steroids, proteins, tannins, resins and saponins using standard qualitative methods as described by Harborne (1973)<sup>36</sup> are given in Table 1.

Physicochemical characters: The powder of leaf was analyzed for various physicochemical constants.

*Organoleptic characters:* The leaf powder was tested with various solvents and chemicals to determine consistency and organoleptic characters which were recorded in Table 2.

*Fluorescence analysis:* The behavior of the powdered leaf in different solutions and their extracts towards ordinary light were observed and recorded in Table 3.

Pharmacological activities:

*Antibacterial activity:* Ethanol, acetone, dichloromethane and petroleum ether extracts of both leaf and stem was performed by using agar well diffusion method at a concentration ranging from 50-250mg/ml were taken and compared with standards Kanamycin (30 µg), Norfloxacin (10 µg) and Ciprofloxacin (5 µg). The bacterial pathogens were strongly inhibited by leaf extracts where as acetone extracts of stem have failed to inhibit the growth even at higher concentration. Therefore the results revealed that leaf extracts were more effective than those of stem extracts. *E. linneanum* possess antimicrobial activity against most commonly encountered human pathogens<sup>37</sup>.

The antimicrobial activity of hexane, ethyl acetate and methanol extracts from the roots of *E. viride* (Forssk.) Alston were determined. The extracts were treated against nineteen bacterial and twelve fungal species using disc diffusion method followed by determination of minimum inhibitory concentration (MIC) by broth dilution method. The results of the study revealed that ethyl acetate extract possesses higher degree of antimicrobial activity than other extracts. The highest antibacterial activity (MIC-0.039 mg/ml) was exhibited against *Staphylococcus aureus* (ATCC 25923), while the highest antifungal activity (MIC-0.25 mg/ml) was exhibited against *Malassezia pachydermatis*. Thus the ethyl acetate extract showing significant antibacterial and moderate antifungal activity could be used in the treatment of infectious diseases<sup>38</sup>.

The ethanolic extract of leaves of *E. linneanum* (Acanthaceae) was assessed for its possible antibacterial activity by disc diffusion method against ten pathogenic bacterial strains. Both Gram negative and Gram positive bacteria at the dose of 250 and 500 µg/disc are compared with positive control Mecillinam (25 µg/disc) by measuring the diameter of Zone of Inhibition in terms of millimeter<sup>39</sup>.

The ethanolic extract of leaves of *E. linneanum* was assessed for its possible antibacterial activity against a number of both Gram negative and Gram positive bacteria but it did not show any effect<sup>40</sup>.

*Antifungal activity:* The antifungal activity of aqueous and methanolic leaf extracts of *E. viride* were tested against four different fungi showed high activity when compared with aqueous extract<sup>41</sup>.

Antifungal property of methanol, ethyl acetate and chloroform leaf extracts of *E. viride* were studied against three different fungi using agar well diffusion method and evaluated by measuring diameter of Zone of Inhibition. Significant antifungal activity was observed in methanol extract of *E. viride* leaves<sup>42</sup>.

From ethyl acetate extract of *E. viride* roots an active compound Ecbolin A was isolated. Minimum Inhibitory Concentration (MIC) of the active compound was studied against twelve fungi using micro-broth dilution technique. The results showed moderate antifungal activity<sup>43</sup>.

*Cytotoxic activity:* Cytotoxicity of the active methanolic extracts of aerial parts was evaluated against the MRC5 human cell line. Cytotoxicity was measured by the colorimetric MTT assay in 96-well microplates. The 50% inhibitory concentration (IC<sub>50</sub>) value was estimated from a dose-response curve. IC<sub>50</sub> (µg/ml) value for the extract was found to be 60.1<sup>44</sup>.

A novel flavone glycoside – luteolin 7-O-(2'' sinapoyl) glycoside was isolated from the ethyl acetate fraction of root of *E. viride* and was screened for *in vitro* cytotoxicity by using Sulfordamine B (SRB) assay method. In this method the compound at five logarithmic dilutions were exposed to 57 human cancer cell lines. The results showed that this flavone glycoside has good cytotoxic activity against human tumor cell lines with an average of 50% growth inhibition in the range of 4.0 – 4.3 µm<sup>45</sup>. Cytotoxicity of ethanolic leaf and stem extracts of *E. linneanum* were performed on the HeLa cervical cancer cell line using the Trypan Blue Dye Exclusion method. The leaf extract exhibited over 80% death of HeLa cells at a treatment concentration of 2mg/ml and stem extract about 78%. The cell viability data obtained in these extracts allow us to predict their potential not only because of the cystostatic effect, but in terms of potential for tumour reduction<sup>46</sup>.

*Antidiabetic activity:* Non insulin dependent diabetes mellitus (NIDDM) was induced in overnight fasted rats by intraperitoneal injection (IP) of 60 mg/kg streptozotocin (STZ) and 120 mg/kg of nicotinamide after 5 min interval. The ethanolic extract of *E. viride* at the dose of 100 and 200 mg/kg and the standard drug glibenclamide (5mg/kg) were administered orally to the diabetic rats for 3 weeks. The FBG levels were estimated on 0 day, 3 days after STZ, for 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> week. The ethanolic extract of *E. viride* 200 mg/kg shows more potent activity than 100 mg/kg compared with standard drug glibenclamide<sup>47</sup>.

*Anti-diarrhoeal activity:* Antidiarrhoeal activity of the ethanolic leaf extract of *E. linnaenum* was tested by castor oil induced diarrhoea in mice. The extract caused a

Table 3. Analysis of fluorescence characters of leaf powder of *E. viride* in different chemical reagents

Sl. No	Chemical reagent	Appearance
1	Powder colour	Pale green
2	5% NaOH	Green
3	10% NaOH	Light green
4	Con. H <sub>2</sub> SO <sub>4</sub>	Light brown
5	Acetic Acid	Light brown
6	1N NaOH in H <sub>2</sub> O	Light green
7	5% KOH	Light green
8	50% HNO <sub>3</sub>	Brown
9	5% FeCl <sub>2</sub>	Dark green
10	1N HCl	Light green
11	Con. HNO <sub>3</sub>	Light green
12	1N NaOH in Ethanol	Green
13	50% H <sub>2</sub> SO <sub>4</sub>	Light green
14	50% HCl	Light green
15	Con. HCl	Light green

significant increase in latent period [1.18 (p<0.005) and 2.04 (p<0.001) hour] i.e. slows the onset of diarrhoeal episode at the doses of 250 and 500 mg/kg of body weight respectively as compared to the standard antidiarrhoeal agent Loperamide where the mean latent period was 2.49 hours. The leaf extract also significantly decreased the frequency of defecation at the doses of 250 mg/kg and 500 mg/kg of body weight where the average mean numbers of stool at the 1st, 2nd, 3rd, 4th hour of study were 6.8 hours and 5.8 hours respectively which was comparable to the standard drug Loperamide, where the average mean numbers of stool at the 1st, 2nd, 3rd, 4th hour of study were 9.8 hours<sup>48</sup>.

Ethanol leaf extracts of *E. linnaenum* were screened for antidiarrhoeal activity by Castor-induced diarrhoea method which showed antidiarrhoeal effect of 6.8 and 5.8 mean inhibition at the doses of 250 and 500mg/kg of leaves extract. It raised the latent period and reduced the number of stools comparing with the standard drug Loperamide<sup>49</sup>.

**Analgesic activity:** Analgesic activity of ethanolic leaf extract of *E. linnaenum* was tested by acetic acid-induced writhing model in mice produced 63.80% writhing at a dose of 250 mg/kg body weight and 45.51% writhing at dose of 500 mg/kg body weight. At the same time the leaf extract produced 36.20% (P<0.01) and 54.48% (P<0.001) writhing inhibition at the doses of 250 mg/kg and 500 mg/kg respectively, which is comparable to the standard drug Diclofenac-Na 75.51% (P<0.001) at the dose of 25 mg/kg<sup>50</sup>.

Ethanol leaf extracts of *E. linnaenum* were screened for analgesic activity by Acetic acid induced Writhing method. The analgesic activity of extract was 54.48% (P<0.001) for 500mg/kg and 36.20% (P<0.01) for 250 mg/kg while standard drug diclofenac inhibition was found to be 75.52% (P<0.001) at a dose of 25mg/kg body weight<sup>51</sup>.

**Antioxidant activity:** The antioxidant potential of methanolic extract of *E. viride* (Forssk).Alston roots was investigated by employing three different established *in vitro* methods, such as DPPH radical scavenging activity, Nitric oxide radical scavenging activity and reducing

power assay. The data obtained in these three methods were found to be 78.25%, 69.79% and 0.2756(absorbance) at 100µg/ml concentration respectively comparable to standard. Results obtained in the present study reveal methanolic extract of *E. viride* (Forssk).Alston possess significant antioxidant activity<sup>52</sup>.

The antioxidant activity of the plant extracts was evaluated by *in vitro* methods for leaf and stem extracts. The DPPH free radical scavenging activity of dichloromethane and ethanolic extracts of leaf and stem exhibited more than 50% whereas ethanol and acetone extracts of exhibited potential effect of 72.7 and 73.1% at 500µg/ml when compared with standard ascorbic acid and all the extracts exhibited lower activity than ascorbic acid. Reducing power assay of extracts were compared with standard ascorbic acid, it was found that acetone extract of stem, ethanol and acetone extracts of leaves exhibited higher activity, whereas activity of petroleum ether extract of leaf was similar to that of ascorbic acid. The total antioxidant potential was higher in acetone extracts of leaf and stem. The lowest antioxidant activity was always exhibited by the petroleum ether extract irrespective of plant part<sup>53</sup>.

Antioxidant activity of methanolic extract of roots of *E. viride* was evaluated by using various *in vitro* methods. The IC<sub>50</sub> values were found to be 33.59 µg/ml in Hydrogen peroxide, 24.37µg/ml in Lipid peroxidation, 26.60 µg/ml DPPH, 68.75µg/ml in Nitric Oxide Radical scavenging, 53.80µg/ml Superoxide Radical scavenging, 49.60µg/ml in Hydroxyl Radical scavenging<sup>54</sup>.

The Percentage inhibition of ascorbic acid and ethanolic extract of *E. linnaenum* leaves were observed. The IC<sub>50</sub> (inhibitory concentration 50%) of ascorbic acid is approximately 1µg/ml and it is more than 500µg/ml for the extract<sup>55</sup>.

**Hepatoprotective activity:** The methanolic extract of whole plant of *E. viride* was tested against carbon tetrachloride and paracetamol-induced hepatotoxicity in rats. *E. viride* (100mg/kg and 200mg/kg) exhibited significant hepatoprotective activity by reducing carbon tetrachloride and paracetamol-induced change in biochemical parameters like SGOT, SGPT, ALP, Total

bilirubin, direct bilirubin and liver glutathione were evident by enzymatic examination<sup>56</sup>.

The methanolic extract of *E. viride* root was evaluated for hepatoprotective effect against CCl<sub>4</sub> induced hepatic injury in rats. CCl<sub>4</sub> (1ml/kg) enhanced levels of biochemical markers like SGPT, SGOT, ALP, triglycerides, liver weight and reduced total proteins significantly. Treatment with methanolic extract of *E. viride* (200mg/kg and 400mg/kg) has brought back the altered levels of biochemical markers to near normal levels in dose dependent manner<sup>57</sup>.

Protective effect of ethanolic and aqueous leaf extract of *E. viride* (Forsk.) Alston on acetaminophen (APAP) provoked hepatotoxicity in rats was investigated. Extracts were administered at the dose of (200 & 400 mg/kg p.o), for 14 days and standard Silymarin was administered. Hepatotoxicity was induced by oral intubation of APAP at the dose of 2g/kg on 14th day of treatment protocol. APAP intoxication resulted in significant increase in the serum hepatic markers like aspartate transaminase, alanine transaminase, alkaline phosphatase, acid phosphatase, -glutamyl transpeptidase, lactate dehydrogenase and total bilirubin. Furthermore, APAP altered the hematological profiles like total leukocyte count, total erythrocyte count and hemoglobin content and lipid parameters. Treatment had significantly ameliorated the toxic events of APAP and maintained the structural integrity of the hepatocytes. *E. viride* may be useful as a potential candidate preventing hepatic damage induced by APAP<sup>58</sup>.

Hepatoprotective activity of ethanolic leaf extract of *E. viride* (Forssk.) Alston in CCl<sub>4</sub> induced rats was evaluated. Rats were treated orally for seven days with suspension of extract (100mg/kg) and (200mg/kg) and reference drug silymarin (25 mg/kg), respectively. Biochemical parameters were estimated. The results indicate that hepatoprotective activity has been confirmed by biomedical parameters and Histopathological studies<sup>59</sup>.

Ethanolic and aqueous extract of leaves of *E. viride* were scrutinized to evaluate protective effect on acetaminophen provoked toxic hepatitis. Treatment with extracts at the dose of (200 & 400 mg/kg), significantly ameliorated the toxic manifestations to normalcy. Treatment with extracts significantly (P < 0.001) counteracted the lipid per oxidation, restored level of Glutathione G, antioxidant enzymes and levels of membrane – bound phosphatases. In conclusion, *E. viride* may be useful as a potential candidate preventing hepatic damage induced by acetaminophen through its antioxidative and lipid peroxidative effect<sup>60</sup>.

The ethanolic extract of *E. viride* roots showed a remarkable hepatoprotective activity against paracetamol-induced hepatotoxicity as judged from the serum marker enzymes in rats. Treatment of rats with different doses of plant extract (100,200 and 400 mg/kg) significantly (p<0.001) altered serum marker enzymes levels to near normal against paracetamol treated rats when compared with standard drug Silymarin (25mg/kg)<sup>61</sup>.

**Anti-trypanosomal activity:** The anti-trypanosomal activity for methanolic extracts of aerial parts of *E. viride* was tested *in vitro* against *Trypanosoma brucei brucei* GUT at 3.1 strain by a dose-response curve using Alamar Blue sensitivity assay. Melarsoprol and pentamidine were used as positive controls. The 50% inhibitory concentration (IC<sub>50</sub>) value estimated from a dose-response curve was found to be 9.37<sup>62</sup>.

**Anti-inflammatory activity:** The anti-inflammatory activity of *E. viride* root extract was evaluated in an *in-vivo* model. The ethyl acetate fraction was administered orally to rats was determined by carrageenan-induced paw edema and cotton pellet granuloma models. Oral administration of *E. viride* extract reduced inflammation significantly (P<0.01) in both the carrageenan paw edema and the cotton pellet granuloma models. The results of the study supported the traditional use of *E. viride* in the treatment of inflammatory disease<sup>63</sup>.

**Anti-Plasmodial activity:** The antiplasmodial activity for methanolic extracts of aerial parts of *E. viride* was tested *in vitro* against chloroquine-resistant strain (K1) and sensitive strain (FCR3). Samples were screened by *in vitro* antimalarial assay system by monitoring parasite lactate dehydrogenase (pLDH) activity using Malstat reagent on strains of *Plasmodium falciparum*. Chloroquine and artemisinin were used as positive controls. The 50% inhibitory concentration (IC<sub>50</sub>) value estimated from a dose-response curve was found to be >12.50<sup>64</sup>.

**Corrosion inhibition studies:** The corrosion inhibition of hexane extract of root and stem of *E. viride* plant on the mild steel corrosion in 1 M HCl has been investigated by weight loss, Tafel polarization and electrochemical impedance spectroscopy (EIS). The corrosion inhibition efficiency increases on increasing the plant extract concentration. Adsorption of inhibitor was found to follow the Langmuir's adsorption isotherm. Thus, it can be classified as anionic inhibitor. Adsorption equilibrium constant (K<sub>ads</sub>) and free energy of adsorption ( *G*<sub>ads</sub>) were calculated and discussed. Surface analysis (SEM) was also carried out to establish the corrosion inhibitive property of *E. viride* in 1 M HCl solution<sup>65</sup>.

**Conflict of interest statement:** We declare that we have no conflict of interest.

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