ISOLATION, CHARACTERIZATION AND EVALUATION FOR ANTIPLASMODIAL ACTIVITY OF EXTRACTED CONSTITUENTS FROM *CAESALPINIA CRISTA LINN* SEEDS

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ABSTRACT

The present work deals with extraction, isolation and characterization of phytoconstituents from seeds of *Caesalpinia crista Linn* and the study of their antiplasmodial activity. The phytoconstituents were extracted using Soxhlet apparatus and separated by column chromatography. Structural elucidation was done by FTIR spectroscopy, NMR spectroscopy and mass spectrometry. Terpenoid, saponin and steroidal glucocorticoid were successfully isolated and identified. These constituents were tested for antimalarial activity using Peter's 4 day suppressive test. Antiplasmodial efficacy was evaluated by parasitemia levels, percentage inhibition, packed cell volume (PCV), survival period, body weight and rectal temperature change. From the ethyl acetate extract, the fractions obtained with a solvent system hexane:chloroform (4:6 V/V), hexane:chloroform (1:9 V/V) and chloroform:ethyl acetate (6:4 V/V) exhibited promising antimalarial activity and particularly first constituent showed significant (P<0.001) antiplasmodial activity than other two phytoconstituents in comparison with positive and negative control.

Keywords: Isolation, characterization, isolates, antimalarial activity, *Caesalpinia crista Linn.*

INTRODUCTION

Caesalpinia crista Linn is a woody scrambling climber belonging to the family *Caesalpiniaceae. Caesalpinia* is utilized in folkloric medicine for treatment of infections, skin diseases, malaria, wounds, fungal infections, fever and pains[1,2]. Cassane diterpenoids are isolated from *C. crista* seeds[3] and norterpenins A and B are isolated from roots and stems[4]. Neocaesalpins H and I or cassane diterpene acids are isolated from leaves[5], Flavonoids, phenolic acids-ferulic acid and gallic acid have been isolated from *C. crista* leaves. *C. crista Linn* was reported for antioxidant activity[6,7], anti-viral activty[8], anticancer activity[9], anti-diabetic activity[10] and antiulcer activity[11]. Since *Caesalpinia in* folkloric medicine was used for the treatment of malaria, a number of compounds have been isolated from various plant parts but no report is available for constituents responsible for antiplasmodial activity. The aim of this work was to isolate active constituents responsible for antimalarial activity from seeds of *C. crista Linn.*

MATERIALS AND METHODS

Chemicals

The solvents, reagents and chemicals used were of analytical grade and were purchased from S.D Fine Chem. Pvt. Ltd., Mumbai, India.

Procurement and authentication of plant material

Caesalpinia crista Linn seeds were collected from local areas of Warangal, Telangana in the month of January 2018, and taxonomically authenticated by Prof. S. Raju, Botanist, Kakatiya University and voucher specimen (vpc/cog/24/18) was deposited (Fig. 1).

Fig. 1: *C. crista Linn* flowers, leaves and seeds

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Experimental animals

Wistar rats were procured from Sainath Labs, Hyderabad. The animals were housed in groups of 6 in cages bedded with paddy husk, fed with commercial pellet diet, given water *ad libitum* and maintained under laboratory conditions (temperature 24 – 28 °C, relative humidity 60 – 70 %, and 12 h light-dark cycle) and were acclimatized for a minimum of 7 days before experiment was performed. Food was with held for 12 h before the start of experiments. The study protocol was approved by the Institutional animal ethical committee (IAEC). (1663/PO/Re/S/12/CPCSEA, 26/04/2017).

Parasite

Chloroquine-sensitive *Plasmodium berghei* strain was obtained from Parasitology Department, Department of Microbiology, Kakatiya Medical College and M.G.M Hospital and was maintained in the laboratory by serial blood passage from mouse to mouse on a weekly basis.

Extraction

Seeds were completely dried and blended to fine powder (300 g) by electrical grinding mill, then extracted for 6 h successively with hexane, ethyl acetate and methanol solvents using Soxhlet apparatus and concentrated to dryness under reduced pressure by applying vacuum to get respective extracts[12].

Phytochemical screening and thin layer chromatography

All the chemical tests and thin layer chromatography were carried out on all the extracts using standard procedures as mentioned in Harbone (1976). Mayer's test for alkaloids, Molisch test for carbohydrates, Borntrager's test for glycosides, Salkowski test for terpenes and sterols, foam test for saponins, Shinoda test for flavonoids, gelatin test for tannins, ferric chloride test for phenols as well as filter paper press test for fats and fixed oils were performed to know the chemical nature of extracts. Small quantities of samples were taken, dissolved in their respective solvents and subjected to TLC analysis using different mobile phases. All plates were visualized under UV cabinet, in the iodine chamber and then Rf values of different spots were calculated. Based on the results, the extracts and solvent systems were selected for column chromatography[13].

Fractionation of extract using column chromatography

The column was packed using dry packing method with 50g silica gel. Based on preliminary phytochemical screening and TLC studies, ethyl acetate extract was selected for fractionation. From the crude ethyl acetate extract, nearly 7 g was adsorbed on silicagel. The adsorbed sample was applied to the top of packed silicagel in column chromatography. The column elution was started initially with hexane, followed by increasing polarity of solvent system, chloroform and ethyl acetate and nearly 60 of each 20 mL fractions were collected. All the 60 fractions were checked for the presence of spot on TLC. The first twenty two hexane:chloroform (9:1 V/V) fractions were colorless and showed no spot on TLC with different solvent systems. Then the fractions F-23, 24, 25, 26, 27, 28 obtained with the solvent system hexane:chloroform in the ratio (4:6 V/V), and F-35, 36, 37, 38, 39, 40, 41, 42 with hexane:chloroform (1:9 V/V) and F-53, 54, 55, 56, 57, 58 with chloroform:ethyl acetate (6:4 V/V) showed spots on TLC plates with chloroform:acetic acid as 9:1 mobile phase for TLC. From these fractions, F-23-28, F-35-42 and F-53-58 were pooled together for spectroscopic analysis taking into consideration the quantity and single spot with same Rf value (purity). TLC analysis was carried out on 0.2mm thickness TLC plates of Merck silica gel 60 F_{254} coated on aluminium plate. Compounds on TLC were detected using UV light[14].

Characterization

FTIR spectroscopy (Bruker, wave number range 4000-400 cm^{-1}), NMR spectroscopy (Bruker, nuclear magnetic field 300 Mega Hz) and mass spectrometry (TOF MS ES+ 174. ABHAY BIOTECH) were utilized for characterization of phytoconstituents[15].

ANTIPLASMODIAL ACTIVITY

Acute toxicity Test

The phytoconstituents were tested in Wistar rats aged 6–8 weeks and weighing 250–300 g. For each phytoconstituent 8 rats were used by randomly dividing them into two groups of 4 rats per group. The rats were starved for 3 h before the experiment commence and only allowed water *ad libitum*. After 3 h, the extracts were orally given in a single dose. The rats in group I were given 0.2 mL of 500 mg kg^{-1} body weight of the extracts. The rats in the control groups received 0.2 mL of the vehicle (dimethyl sulfoxide) used for dissolving the extracts. Then, the rats were observed continuously for 1 h, followed by 4 h observations for 24 h and thereafter daily for 14 days, for any manifestation of toxicity[16-18].

Dilutions of drugs and test samples

Stock solutions of isolates were prepared in dimethyl sulfoxide (DMSO), while chloroquine stock solution was prepared in water (Milli-Q grade).

Parasite inoculation

Albino rat earlier infected with *Plasmodium berghei,* ANKA strain, (parasitaemia level of 20–30%) was used as donor. The donor rat was then sacrificed with chloroform anesthesia and blood was collected by cardiac puncture into heparinized vacutainer tube. The blood was then diluted with physiological saline (0.9 %) based on parasitaemia level of the donor rats and the red blood cell (RBC) count of normal rats, in such a way that 1 mL blood contains 5×10^7 infected RBC . Each rat was then given 0.2 mL of this diluted blood intraperitoneally, which contained 1×10^7 *P. berghei* infected RBCs[19].

Four-day suppressive test

Antiplasmodial activity of the test isolates were evaluated in a 4 day suppressive standard test. Wistar rats weighing 25–29 g were inoculated with 0.2 mL of infected blood on the first day (day 0), intraperitoneally. The rats were then divided randomly into five groups of six rats per group. Two groups (II and III) were assigned as test groups whereas the other two groups (I & IV) were used as control (negative and positive) groups. 3 h after infection 15 mg $kg^{-1}day^{-1}$ and 25 mg $kg^{-1}day^{-1}$ of three isolates *C. crista Linn 1 (C.c 1), C. crista Linn 2 (C.c 2) and C. crista Linn 3 (C.c 3)* were administered to the test groups. Chloroquine at the dose of 25 mg $kg^{-1}day^{-1}$ and an equivalent volume of vehicle (0.2 mL 7% dimethyl sulfoxide solution) were administered to the positive and negative control groups, respectively, for four consecutive days (day 0–3). On the fifth day (on day 4, 24 h after the last dose i.e. 96 h post-infection), thin blood smears were made from the tail of every rat, fixed with methanol and stained with 10% Giemsa. The parasitaemia level was determined by counting the number of parasitized erythrocytes out of 100 erythrocytes in random 8 fields of microscope.

Average percentage suppression was calculated as 100[A–B/A].

where A, represents average percentage parasitaemia in control group and B is average percentage parasitaemia in the test group. The capability of the various doses of the crude isolates in preventing body weights and packed cell volume (PCV) reduction of the rats as a result of raise in parasitaemia were followed. The weight, PCV and rectal temperature and mean survival period were also observed[20-23].

Statistical analysis

Data were analysed using Graph Pad Prism v 6.02. Comparisons were made among negative and positive controls as well as treatment groups using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Mean PCV, rectal temperature and body weight before and after infection and treatment were compared by two tailed paired *t* test. The data were expressed as mean ± standard deviation (SD). The result was considered statistically significant at 95 % confidence level and $P < 0.001$.

RESULTS AND DISCUSSION

Extraction

3 g of yellow semisolid hexane extract, 8 g of brownish red semisolid ethyl acetate extract and 4 g of creamy yellow semisolid methanolic extract were obtained.

Phytochemical screening and thin layer chromatography

The plant *C. crista Linn* shows the presence of carbohydrates, saponins, alkaloids, steroids, glycosides and triterpenoids. The TLC of ethyl acetate extract showed the presence and separation of three spots with Rf values 0.08, 0.25 and 0.6.

Fractionation of extract

Ethyl acetate extract was selected based on preliminary phytochemical studies and TLC profile which was subjected for column separation using silica gel as stationary phase, solvents of increasing polarity as solvent system for column and chloroform:acetic acid (9:1 V/V), ethyl acetate:acetic acid (9:1 V/V) as mobile phase for TLC of isolated constituents. Three greenish yellow, light brown and cream coloured crystalline compounds were obtained by column chromatography in ethyl acetate extract with solvent system (chloroform:hexane (6:4 V/V), hexane : chloroform (1:9 V/V) and chloroform:ethyl acetate (6:4 V/V) .

Characterization

First constituent *C. crista Linn*-1 was found to be steroidal nucleus glucocorticoid by analysis of spectral data from IR spectroscopy, NMR spectroscopy and mass spectrometry. The IR spectrum showed bands at aliphatic C-H stretch (2927.80 cm^{-1}, aromatic C- H stretch (3000 cm^{-1}) , C-O stretch (1017.88 cm^{-1}), C=O stretch (1733.60, 1800 cm^{-1}), aliphatic CH bends (1452.34, 1378.22cm^{-1}). The ^1H NMR spectra showed the presence of chemical shift ranges (ppm) for types of protons R-CH_3(10-25), R_2CH_2 (20-40 ppm), R_3CH (45-55 ppm), C=O (79 ppm), benzene (127-133 ppm) and ^{13}C -NMR spectrum indicated the presence of chemical shift ranges (ppm)for types of

carbons R-CH_3 (10-25 ppm), R_2CH_2 (20-40 ppm), R_3CH (45-55 ppm), C=O (79 ppm), benzene (127-133 ppm). From the mass spectra based on the positive ion MS [*m*/*z* 378(M+H)] analysis the molecular weight of *C.c* 1 is found to be 378. The molecular formula of first constituent is $C_{27}H_{38}O$ based on nitrogen rule - even molecular weight may be even nitrogen atoms are present or no nitrogen atoms ($C_aH_bN_cO_d$) and ring rule R = a+1+(c-b)/2,R = 27+1+0-38/2=28-19=9 where R=No of rings +no of double bonds +no of triple bonds=4+5. Therefore, the constituent-1 is cyclopenteno perhydro phenanthrene nucleus, steroidal nucleus glucocorticoid.

Second constituent *C. crista Linn* -2 is found to be steroidal saponin by spectral analysis. The IR spectrum showed the presence of bands at aliphatic CH (2857.44cm^{-1}), CN stretch (2311.68cm^{-1}), long chain out of plane bending ((757.58cm^{-1}), aliphatic – CH bends (1452.34, 1376.22cm^{-1}). The ^1H NMR spectrum showed the presence of chemical shift ranges (ppm) for types of protons as R-CH_3 (0.83 -0.9 ppm) , R_2CH_2 (0.9 -1.6 ppm), R_3CH (1.6 -2 ppm), CH-F (4.3- 4.6 ppm). The ^{13}C NMR spectrum showed the presence of chemical shift ranges (ppm) for types of carbons as R-CH_3 (10-25 ppm), R_2CH_2 (20-40 ppm), R_3CH (45-55 ppm) and C-X (77-80 ppm). The molecular weight of *C. crista Linn* 2 was found to be 349 based on the positive ion LC-MS [*m*/*z* 349(M+H)+] analysis. The molecular formula is shown to be $C_{23}H_{37}FO$ based on nitrogen rule - even molecular weight may be even nitrogen atoms are present or no nitrogen atoms and, ring rule $C_aH_bN_cO_d$ R = a+1+(c-b)/2 where R = 23+1+0-37/2=24-19=5, R=No of rings +no of double bonds +no of triple bonds. Therefore, the second constituent is cyclopenteno phenanthrene nucleus i.e., a steroidal saponin.

The third constituent of *C. crista Linn-3* is found to be a terpenoid. The FTIR spectra showed bands at aliphatic C-H (2927cm^{-1}), aliphatic C-H bends (1452.34-1378cm^{-1}) and long chain out of plane bending CH bends (-757.58cm^{-1}). The ^1H NMR spectrum showed the presence of chemical shift ranges (ppm) for types of protons as R-CH_3 (10-20 ppm), R_2CH_2 (0.9 -1.6 ppm), R_3CH (1.6 -2 ppm) and benzene 7.2 - 8. The molecular weight of *C. crista Linn* 3 was found to be 441 based on the positive ion LC-MS [*m*/*z* 441(M+H)+] analysis. The molecular formula is shown to be $C_{31}H_{52}O$ based on nitrogen rule - even molecular weight may be even nitrogen atoms are present or no nitrogen atoms. $C_aH_bN_cO_d$ and ring rule R = a+1+c-b/2: R = 31+1+0-52/2=32-26=6, R=No of rings +no of double bonds +no of triple bonds=6. In conclusion, the third constituent is a terpenoid (Fig. 2).

ANTIPLASMODIAL ACTIVITY

Acute toxicity studies

In the *in vivo* acute toxicity assessment of the isolates, there were no gross and physical changes such as rigidity, depression, abnormal secretion, sleep, diarrhoea, and hair erection for 24 h. All the rats survived within the 2-week observation period.

Four-day suppressive test

The percentage parasite suppression of *isolates* produced significant reduction in parasitemia levels ($P < 0.05$) at two doses as compared to the negative control group. The group *C. crista Linn* 3 which received 25 mg kg^{-1} exhibited maximal highest percentage parasite

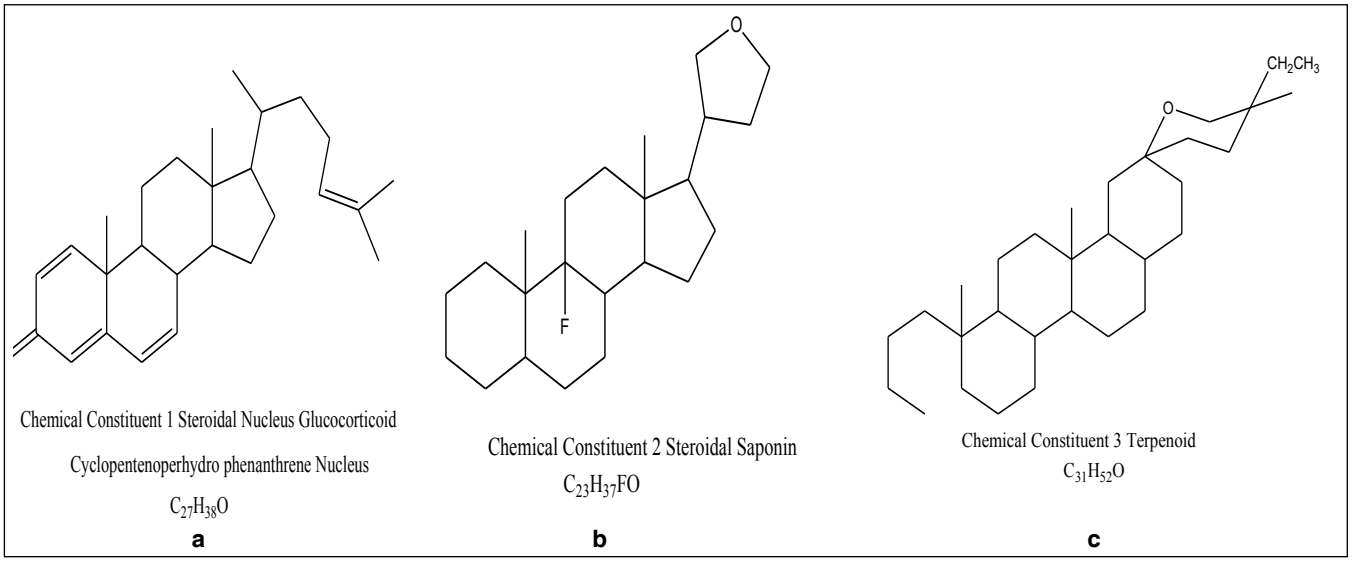

Fig. 2 (a, b, c): Structures of 3 isolated phytoconstituents

inhibition activity *on P. berghei* infected rats as compared to the Control and the standard treatment chloroquine (CQ). Significant difference: P < 0.001 (84.69%) followed by *C. crista Linn* 2 (25 mg kg^{-1} (81.17%) and *C. crista Linn* 1 (25 mg kg^{-1} (79.21%)). The three isolates significantly prolonged the survival time of the rats, prevented significant (p<0.05) body weight reduction, loss of PCV, loss of body temperature on day four as compared to day one in a dose dependent way as compared to the control group. The standard drug CQ25 demonstrated the maximum effect on parasitemia levels, significant P<0.001 percentage parasite inhibition (91.29 %) and survival period. The preventive effect of haematological abnormalities (PCV reduction), body weights loss and rectal temperature drop by the isolates infer its antimalarial activity. Terpenoids constitute the largest class of biologically active product and play a defensive role against predators, pathogens and competitors, maintaining antagonistic and beneficial interactions among organisms. Thus, the presence of terpenoid is attributed for antiplasmodial activity in *C. crisa.* (Figs. 3, 4, 5).

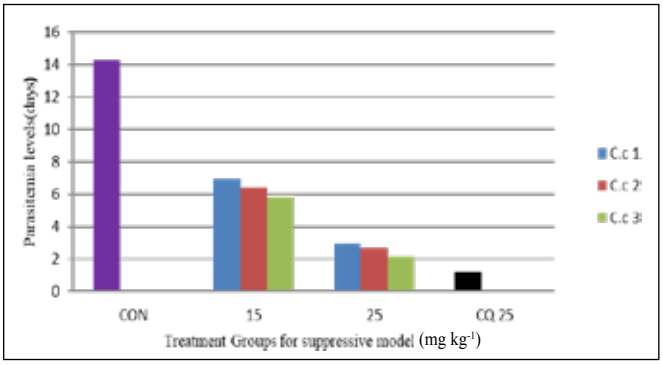

Fig. 3: Four day parasitaemia levels of different doses of isolates in *P. berghei* infected rats as compared to the control (CON) and the standard treatment, chloroquine (CQ) Each bar represents the Mean ± SD for each group of rats, n =6. Significant difference: P < 0.001

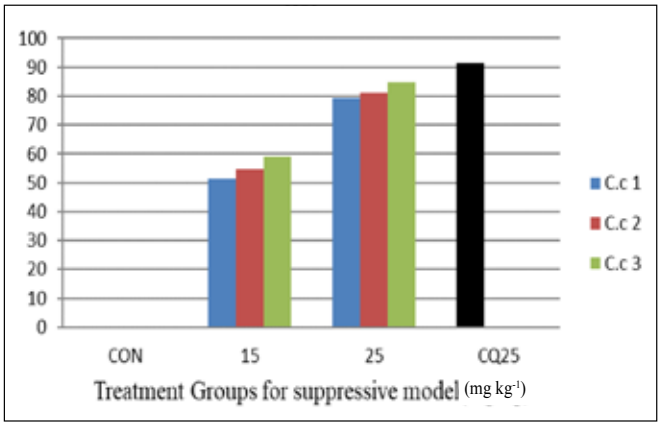

Fig. 4: Percentage parasitemia suppression of isolates on *P. berghei* infected rats as compared to the Control and the standard treatment chloroquine (CQ). Each bar represents the Mean ± SD for each group of rats, n =6

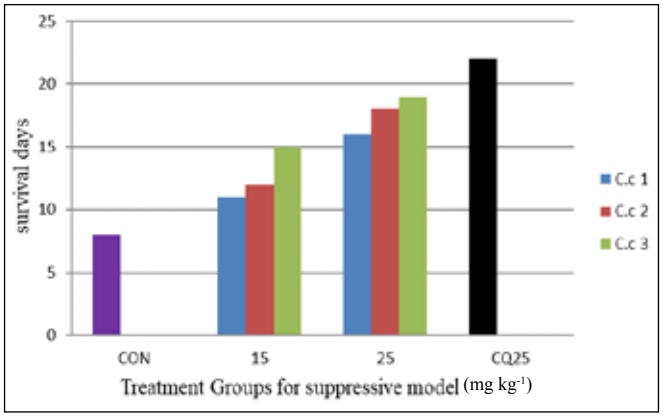

Fig. 5: Mean survival time of isolates as compared to the control and the standard treatment chloroquine (CQ). Significant difference P < 0.001

CONCLUSION

Two previously undocumented glucocorticoid and steroidal saponin with known terpenoid constituents were isolated from the seeds of *C. crista*. All the three phytoconstituents showed significant antiplasmodial activity. Dose dependent increase in activity was observed and at high concentration, the activity is comparable to standard. The terpenoid constituent exhibited promising antimalarial activity compared with the other two constituents.

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