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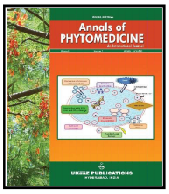

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HPTLC fingerprinting of various extracts of *Couroupita guianensis* flowers for establishment of *in vitro* antimalarial activity through isolated compound

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Abstract

Malaria is the most common infectious disease, due to multi drug resistance parasites and the limited number of effective drugs available in this situation is complicated, it is necessary to discover new antimalarial drugs. HPTLC fingerprint analysis is the most potent tool for quality control and standardization of herbal drugs. In the present study, five different extracts of *Couroupita guianensis* Aubl. flowers were screened for their anti-malarial activity by *in vitro* inhibition of *β* hematin formation assay and their HPTLC fingerprinting was carried out. The most potent antimalarial activity was shown by ethyl acetate extract of flowers then isolation of compound responsible for activity was carried out and characterized by UV, FTIR, H-NMR, Mass Spectroscopy, the structure was found to be Stigmasterol.

1. Introduction

Malaria is a fatal tropical disease, causes by a genus *Plasmodium* (Protozoa). It is a parasite named *Plasmodium falciparum*, affect humans by far the most virulent. As per WHO, the estimated death rate due to malaria was 405 000 in the year 2018 and still the death is occurs globally in several countries (WHO, 2019). There are many synthetic medicines applied for the treatment of malaria but are having many side effects and also shows resistance. Therefore, it is a serious challenge due to the emergence of *P. falciparum* strains resistant to all antimalarial drugs. To overcome the challenge, medicinal plants have played an important role in malaria treatment with their new chemical compounds. It was reported that two major drugs, quinine and artemisinin that are used worldwide, herbal based compounds. Hence, many developing countries are depending on medicinal plants to meet their primary health care needs (WHO, 2010).

Of late, *Couroupita guianensis* Aubl. (CG) commonly called as cannon ball tree, belongs to the angiosperm family Lecythidaceae. This plant has enormous therapeutical uses. In Indian system of medicine, it is used as an ingredient in many preparations to cure gastritis, bleeding piles, dysentery, scabies and many other conditions (Shah *et al.*, 2012; Manimegalai *et al.*, 2014). More than 30 species are recognised for genus *Couroupita* throughout the world. It is native to South India and Malaysia. Cannon ball flowers (Nagalingam flowers) are announced as the State flower by Puducherry Government (Ramalakshmi *et al.*, 2014; Chavda, 2015).

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Characteristics of the plant: CG is a large deciduous tree with height up to 20 meters. Leaves are up to 20 cm long, serrate margin, arranged alternate with oblong to obovate shape. It shows large branches with racemose inflorescence. Flowers are zygomorphic with high fragrance. Flowers are up to 6 cm in diameter with six petals, colour ranges from shades of pink and red with yellow tinge. Stamens are at two areas; a ring of stamens at the centre and stamens borne on an overarching androphore. Fruits are large globose with woody shell hangs in cluster like balls hanging in a string. The fruits have unpleasant smelling edible jelly and small 200-300 white seeds embedded in pulp. Pulp oxidizes to bluish green colour when exposed to air. The hard woody shells of the fruits are used to prepare containers and utensils (Regina, 2014; Aravind *et al.,* 2017; Ramadoss, 2017). Because of its attractive flowers and fragrance, it is grown as a decorative tree in Indian gardens. It is called in different names in different languages like Kailaspati in Hindi, Mallikarjuna flowers in Telugu, Nagalingam flowers in Tamil.

Ethnic uses: In India usually, these trees are planted near Shiva temple and used for Shiva pooja because of its Shivalingam shape present at the centre of the flower and like snake hood designed pollens (Sundararajan and Koduru, 2014). Leaves of CG are used in the treatment of skin diseases (Sumathi and Anuradha, 2017). Leaves and flowers are used in the treatment of cold, enteric gas formation and abdomen ache (Elumalai *et al.*, 2012; Sundararajan and Koduru, 2014). The flower of CG is evident of analgesic, anti-inflammatory, immunomodulatory, anthelmintic, antimicrobial, wound healing, antioxidant and antinociceptive activities (Geetha *et al.*, 2005; Umachigi *et al.*, 2007; Pradhan *et al.*, 2008; Bhagyasri *et al.*, 2014).

Phytochemistry: Phytochemical screening showed the presence of alkaloids, flavonoids, sterols, glycosides, tannins, triterpenes, isatin, indirubin, couroupitine, α-amirin, β-amirin in the leaves and flowers

(Rane *et al*., 2001; Eknat and Shivchandraji, 2002; Gousia *et al*., 2013) and the various therapeutic activities are observed due to the presence of these essential phytoconstituents. The leaves and fruits of CG tree are used to treat malaria by South Americans (Sathishkumar *et al*., 2016; Jasmine and Moorthi, 2017; Sumathi and Anuradha, 2017). Leaves of CG showed good antiplasmodial activity (Kaushik *et al*., 2015) whereas traditionally flower is effective against high fever, abdominal pain, diarrhoea, dysentery, and acts as immune booster (Ramalakshmi *et al*., 2013; Prabhu and Ravi, 2017; Lawrence and Venkatraman, 2020). The symptoms of antimalarial activity and the traditional applications of flowers are quite similar and even there is no scientific evidence for antimalarial activity of CG flowers. Therefore, it was essential to investigate the antimalarial activity of CG flowers and to isolate the compound responsible for the same.

2. Materials and Methods

2.1 Materials

Fresh flowers of CG were collected from T. Dasarahalli, Bengaluru. Identification and authentication of plant material was done by Dr. Rama Rao at Regional Ayurveda Research Institute, Bengaluru. The authentication reference number bears SMPU/RARIMD/BNG/ 2018-19/1580.

2.2 Methods

2.2.1 Extraction of crude drugs

Fresh flowers were collected from Bengaluru during the month of June, cut into small pieces and shade dried for 7-8 days then ground into a coarse powder, sieved using sieve #12.

Dried and powdered flowers of CG (50 g) were extracted by packing into a thimble and extracted in Soxhlet apparatus successively with solvents of increasing polarity, *viz.*, pet-ether, chloroform, ethyl acetate, ethanol, methanol, and water until the siphon tube was colourless. Before extracting with the next solvent, marc was pressed to remove the residual solvent. The extract obtained was filtered and concentrated to dryness. The percentage yield was calculated in terms of air-dried weight of plant material and dried extracts were stored in airtight glass container for further phytochemical screening and antimalarial study.

2.2.2 HPTLC fingerprinting

2.2.2.1 Sample preparation: All extracts were re-dissolved in 1 ml of respective solvents

2.2.2.2 Application of sample: Samples were applied on pre-coated silica gel 60F 254 aluminium sheets (10×10 cm) with the help of Linomat 5 applicator attached to the CAMAG HPTLC system, which was programmed through WINCATS software. The application of bands of each extract was carried out 6 mm band in length and 2 µl in concentration.

2.2.2.3 Developing solvent system: A number of solvent systems were tried for flowers and fruits extract, but the satisfactory resolution was obtained in the solvent system Toluene: Ethyl acetate: Formic acid: Methanol (5.5:3:1:0.5) for flowers extracts.

2.2.2.4 Development of chromatogram: After the application of the sample, the chromatogram was developed in Twin trough glass chamber 20×10cm with solvent system Toluene: Ethyl acetate: Formic acid: Methanol (5.5:3:1:0.5) for flower extracts.

2.2.2.5 Detection of spots: The air-dried plates were placed in the TLC scanner at position 0 (right hand side). The first track spotted should be placed at the position 0 and the slit dimension was adjusted for 5.00×0.45 mm. The chromatograms were scanned by a densitometer at 254 nm and 366 nm in densitometry TLC Scanner 3. The R_f values and fingerprint data were recorded by WINCATS software (Shivatare *et al.*, 2013).

2.2.3 *In vitro* inhibition of β-hematin formation assay

Briefly, varying concentrations of the extracts from 0 to 2 mg/ml in Dimethyl sulfoxide (DMSO) were taken in clean Eppendorf's tube then incubated with 3 mM of hematin (freshly dissolved in 0.1M NaOH), 10 mM oleic acid and 1 M HCl. The volume was adjusted to 1 ml using sodium acetate buffer at pH 5. Chloroquine diphosphate was used as a positive control. The reaction mixtures were incubated overnight at 37°C with frequent shaking and then centrifuged (14000 rpm, 10 min, at 21°C) and the hemozoin pellet was repeatedly washed with incubation (15 min at 37°C) with regular shaking in 2.5% (w/v) sodium dodecyl sulphate in phosphate-buffered saline followed by a final wash in 0.1 M sodium bicarbonate until the supernatant was clear (usually 3-8 washes). Finally, the supernatant was removed by washing and then the pellets were dissolved in 1 ml of 0.1 M NaOH before determining the hemozoin content by measuring the absorbance at 400 nm (Mojarrab *et al.*, 2014; Asnaashari *et al.*, 2015; Afshar *et al.*, 2018). The percentage inhibition (I%) of heme crystallization were recorded and then compared to the positive control, using the following equation:

$$I\% = [(AN-AS) / AN]\times 100$$

where,

AN: absorbance of negative control;

AS: absorbance of the test sample.

2.2.4 Isolation of compound responsible for antimalarial activity

Based on the higher activity against malaria, the particular extract was further subjected to column chromatography on silica gel (#60-120 mesh). 5 g of ethyl acetate fraction was dissolved in 5ml of ethyl acetate and then mixed with 10 gm of silica gel (#60-120) in the ratio 1:2 (a drug to silica gel) and air-dried at room temperature. To a column of 500 ml capacity packed with silica gel, 15 g of silica gel drug mixed material was charged and eluted with hexane (100) [FR 1], hexane: ethyl acetate (80:20) [FR 2], hexane: ethyl acetate (60:40) [FR 3], hexane: ethyl acetate (40:60) [FR 4], hexane: ethyl acetate (20:80) [FR 5], ethyl acetate (100) [FR 6]. The fractions were collected and concentrated over a hot plate. The concentrated fractions were evaluated for *in vitro* antimalarial activity by inhibition of β-hematin formation assay. FR 3 shown maximum activity therefore it was subjected to preparative TLC using mobile phase chloroform: methanol (7:3), which show two spots with Rf value 0.35 and 0.41. The spots were scooped and subjected to antimalarial activity. The isolated compound was characterized by UV, IR, H-NMR, and mass spectroscopy.

2.2.5 Correlation study

Percentage inhibition by the extract was correlated with the percentage yield of the extract and the result was recorded.

Statistical analysis

Data are expressed as Mean ± SEM of triplicates. The concentration showing a higher percentage of inhibition was subjected to statistical analysis with one-way ANOVA, *i.e.*, Dunnett's multiple comparison test to determine the significance of the experimental results.

3. Results

3.1 Extraction and percentage yield of the extract

Various solvents, *viz.,* pet-ether, chloroform, ethyl acetate, ethanol, methanol, and water were used for CG flower extraction and resulted in higher yield (25.3%) in ethyl acetate solvent (Figure 1) and further pharmacognostic screening through chemical tests were performed and the result was shown in Table 1.

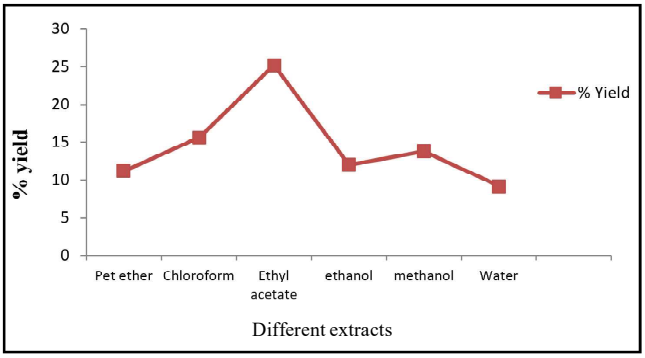

Figure 1: Percentage yield of extract in various solvent.

Table 1: Phytochemical analysis of CG flower extracts

Phytoconstituents	Various tests	PEE	CE	EAE	EE	ME	AE
Alkaloids	Mayer's Test	—	+	—	+	+	—
	Dragendroff's Test	—	+	+	—	+	—
	Wagner'sTest	—	+	—	—	+	—
	Hager's Test	—	+	+	—	—	—
Flavonoids	Shinado Test	—	—	++	—	—	+
	$FeCl_3$ Test	—	—	+	—	—	+
	Lead acetate test	—	—	+	—	—	—
Glycoside	Keller killiani Test	—	—	++	—	—	+
steroids	Salkowski Test	—	—	++	+	+	+
	Liebermann-Burchard Test	—	+	++	—	+	—
Tannins	$FeCl_3$ Test	—	+	++	—	+	+
	GelatinTest	—	+	++	+	+	+
Phenols	FC reagent Test	—	++	++	—	+	+
Proteins	Biuret Test	+	—	—	—	—	—
Saponins	Foam Test	—	+	+	—	—	+
Triterpenes	Salkowski Test	—	+	++	—	—	+
	Liebermann-Burchard Test	—	+	++	—	+	+

- PEE=Pet ether extract; CE=Chloroform extract; EAE=Ethyl acetate extract; EE=Ethanol extract; ME=Methanol extract; AE=Aqueous extract.

3.2 HPTLC analysis

Based on the chemical test report, further study was carried out using HPTLC for five different extracts except for pet ether extract.

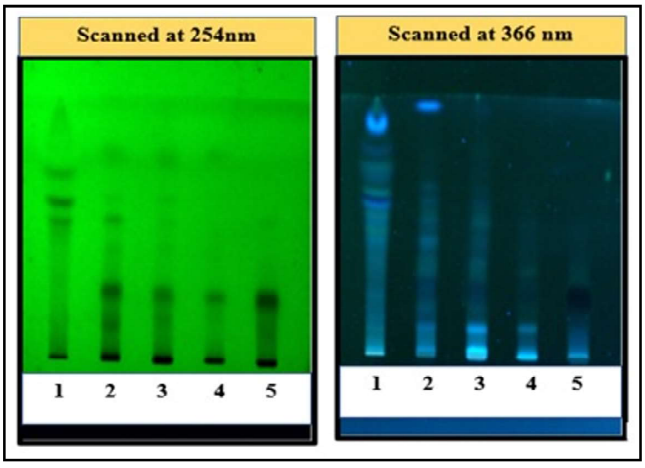

Figure 2: TLC plates scanned at 254 and 366 nm for *C. guianensis* flower extracts Tracks: 1-chloroform extract, 2-ethyl acetate extract, 3-ethanol extract, 4-methanol extract, 5-water extract.

The number of solvent systems were tried for CG flower extracts, but the satisfactory resolution was obtained in the solvent system Toluene: Ethyl acetate: Formic acid: Methanol (5.5:3:1:0.5) for flower extracts. HPTLC chromatogram of different extracts of CG flower at 254 and 366 nm showed in Figure 2 and 3D chromatogram of the extracts showed in Figure 3.

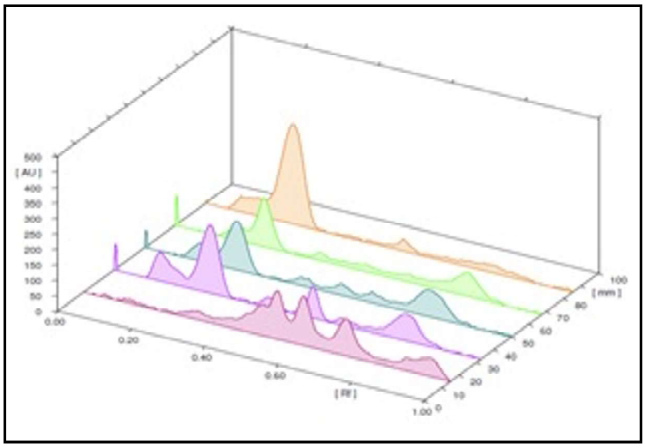

Figure 3: 3D chromatogram of various extracts of CG flowers.

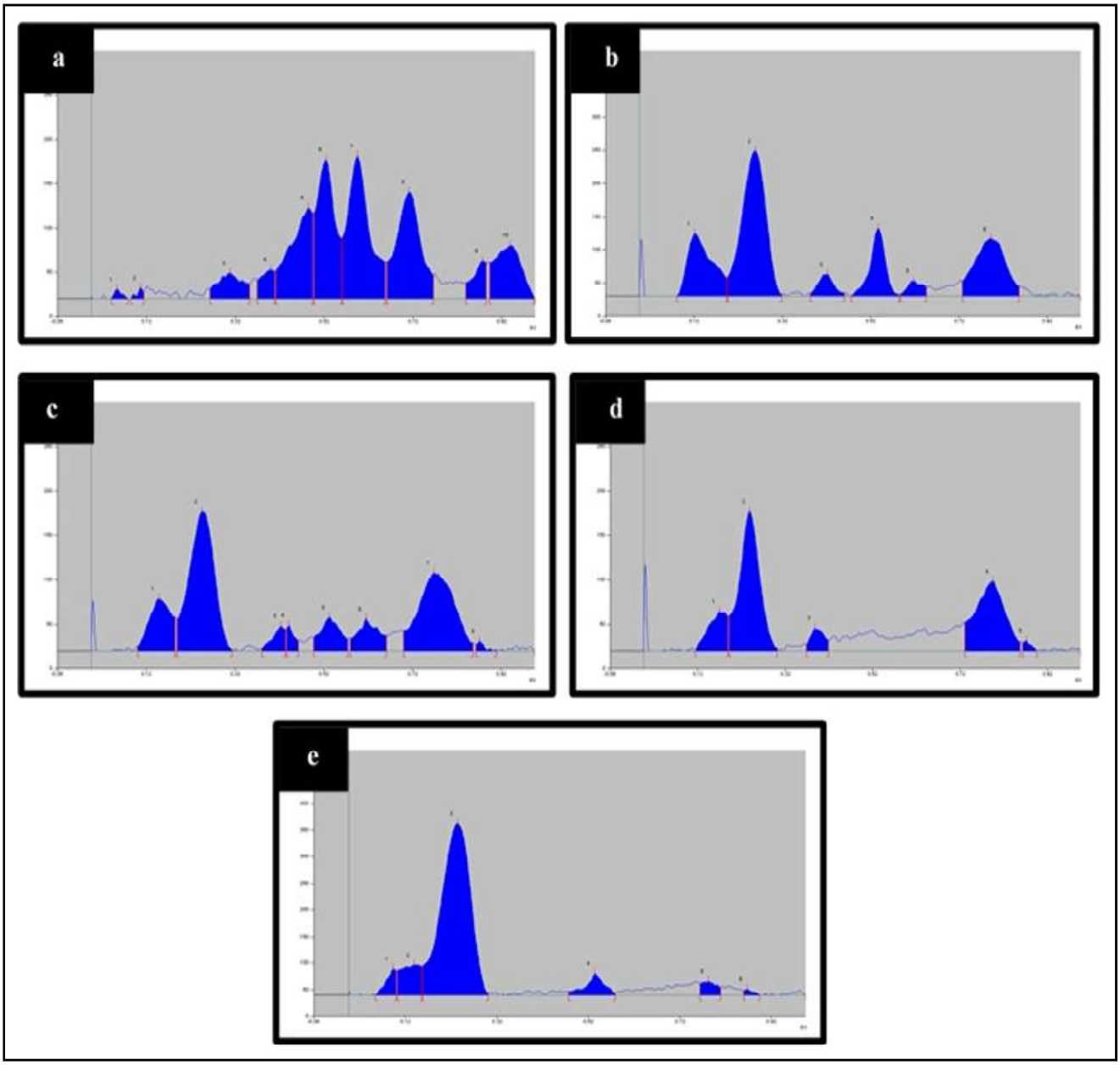

Figure 4: HPTLC chromatogram for various solvent systems of *C. guianensis* flowers; a-chloroform extract, b-ethyl acetate extract, c-ethanol extract, d-methanol extract, e-water extract.

Rf of different extracts of CG flower showed in Table 2.

Table 2: Rf values of various extracts of CG flowers

Sample	Solvent system	No. of peaks	Rf values
Chloroform extract		10	0.06, 0.11, 0.31, 0.40, 0.49, 0.53, 0.60, 0.72, 0.88, 0.94
Ethyl acetate extract	Toluene: Ethyl acetate:	6	0.13, 0.26, 0.43, 0.54, 0.62, 0.80
Ethanol extract	Formic acid:	8	0.15, 0.25, 0.43, 0.45.0.54, 0.62, 0.77, 0.88
Methanol extract	Methanol (5.5:3:1:0.5)	5	0.17, 0.24, 0.39, 0.80, 0.88
Water extract		6	0.1, 0.14, 0.24, 0.54, 0.79, 0.87

3.3 Antimalarial activity

All the extracts of CG flowers were screened for anti-malarial activity by inhibition of beta hematin formation assay as per the procedure mentioned in methodology. Ethyl acetate and chloroform extract of CG flowers showed activity during *in vitro* inhibition of β-hematin formation assay (Table 3).

Table 3: Percentage inhibition of beta hematin formation of various extracts of CG

Sample	0.25 mg/ml	0.5 mg/ml	1.0 mg/ml	1.5 mg/ml	2.0 mg/ml
Chloroform extract	7.20 ± 0.12	13.66 ± 0.26	26.00 ± 0.59	36.60 ± 1.05	47.13 ± 0.35
Ethyl acetate extract	13.8 ± 0.32	24.7 ± 0.75	43.4 ± 0.75	64.43 ± 0.67	77.27 ± 0.27
Ethanol extract	-	-	-	-	-
Methanol extract	-	-	-	-	-
Water extract	-	-	-	-	-
Chloroquine	74.97 ± 0.24 (0.2 mg/ml)				

The samples were tested in various concentrations, activity increases as the increase in concentration for ethyl acetate and chloroform extract of CG flowers and results of concentration showing a higher percentage of inhibition (77.27%) (were subjected to statistical analysis with one-way ANOVA, *i.e.*, Dunnett's multiple comparison test to determine the significance of the experimental results.

Table 4: One-way ANOVA followed by Dunnett's test for antimalarial

Sl. No.	Sample	Concentration	Percentage inhibition
1	Chloroform extract of flowers	2 mg/ml	47.13 ± 0.35**
2	Ethyl acetate extract of flowers	2 mg/ml	77.27 ± 0.27***
3	StandardChloroquine diphosphate	0.2 mg/ml	74.97 ± 0.24

- Values are expressed as Mean ± SEM (n=3) and P** <0.01, and P***< 0.001, significant.

3.4 Isolation of compound

Ethyl acetate extract of CG flowers showed the maximum activity, therefore, this extract was subjected for column chromatography eluting with mobile phase Hexane (100) [FR 1], Hexane: Ethyl acetate (80:20) [FR 2], Hexane: Ethyl acetate (60:40) [FR 3], Hexane: Ethyl acetate (40:60) [FR 4], Hexane: Ethyl acetate (20:80) [FR 5], Ethyl acetate (100) [FR 6]. All six fractions were collected from the Column. The fractions were concentrated and dried under vacuum and evaluated for *in vitro* antimalarial activity by inhibition of β-hematin formation assay. FR 2 showed 36.58 % inhibition whereas FR 3 shown % inhibition of 79.23%. FR 3 was further purified by preparative TLC using mobile phase chloroform: methanol (7:3) which shown 2 spots of Rf value of 0.35 and 0.41. The spots were scooped and evaluated for antimalarial activity. The compound of Rf value 0.41 shown maximum activity 67.23% and it was characterized.

The isolated compound was identified by UV spectrophotometer where λ max showed at 257 nm and the presence of conjugation as well as chromophore. Further, FTIR, H-NMR, and MASS spectroscopy analysis revealed the isolated compound was Stigmasterol (Figure 5, 6, 7 and 8).

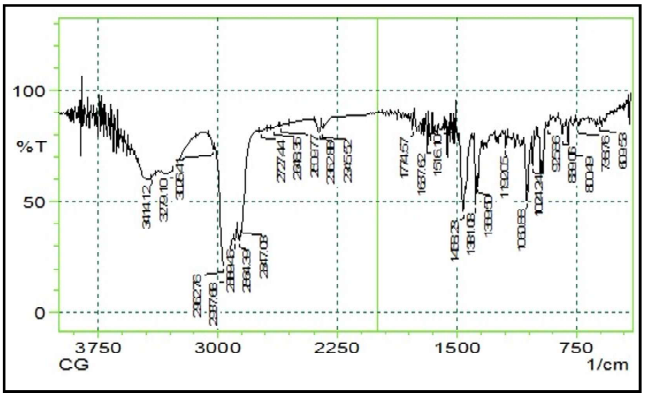

Figure 6: FTIR spectra of isolated compound.

FTIR graph showed the characteristic absorption band were exhibited at 3414.12 cm^{-1}(OH group), 2924.18 cm^{-1} (C-H stretching), 2852.81 cm^{-1} (C-H stretching), 1458.23 cm^{-1} (C-H bend), 1637.62 cm^{-1} (C=C)1192.05 cm^{-1} (OH bending), 1060.88 cm^{-1} C-C stretching), 738.76 cm^{-1} (CH_2 rocking).

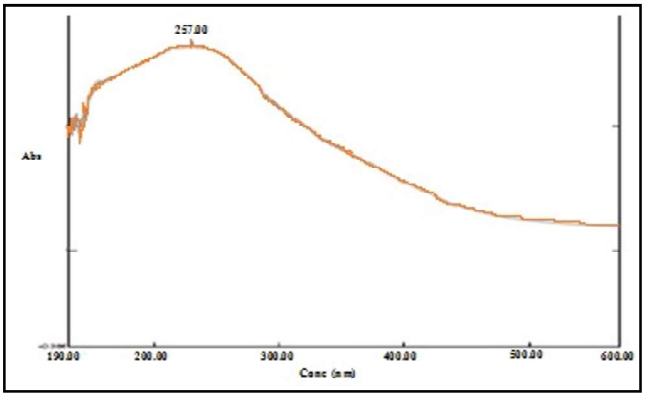

Figure 5: UV absorbance of isolated compound.

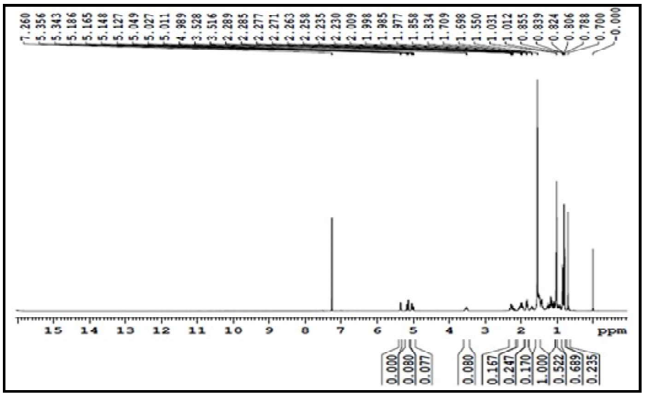

Figure 7: ^{1}H-NMR spectra of isolated compound.

Proton NMR data revealed isolated compound has CH_3 group with chemical shift δ 0.700, CH_2 group was identified with chemical shift δ 0.788-0.855, CH=CH group with chemical shift δ 1.550 and OH group with chemical shift δ 3.489-3.556.

Finally, the MS screening and confirmation of isolated compound was detected by their molecular ions at m/z 412 which correspond to the molecular mass of Stigmasterol 412.69 g/ml.

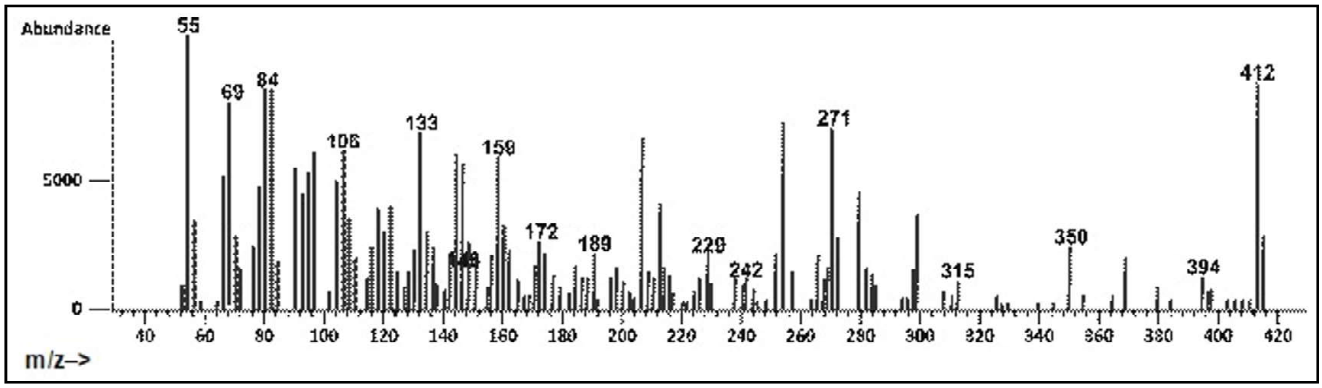

Figure 8: Mass spectra of isolated compound.

All the above data was compared to the values from literature and it is clearly suggested that isolated compound have structure of Stigmasterol (Figure 9).

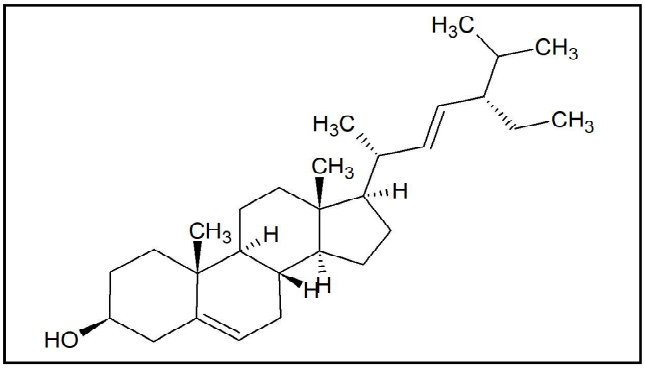

Figure 9: Stigmasterol.

3.5 Correlation study

Based on the activity, ethyl acetate flower extract of CG was correlated with the inhibition of the β-hematin formation and recorded significant positive regration effect with the percentage yield (Figure 10), with the $R^2 = 0.991$.

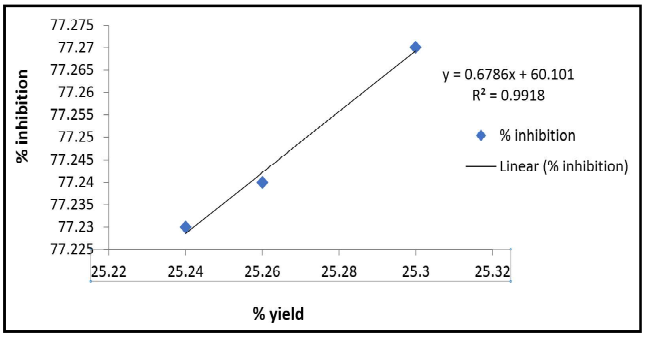

Figure 10: Correlation study of % yield and % inhibition of ethyl acetate extract of CG flower.

4. Discussion

The *Plasmodium* parasite, degrades haemoglobin (Hb) within the infected erythrocytes to use the catabolic products as the source of nutrition for its growth and proliferation. Further, free Hb is released as a toxic byproduct in this process that affects the cellular metabolism with the mechanism of membrane peroxidation and thereafter, inhibits a variety of enzymes. Hence, the malaria parasite uses several detoxification pathways to get rid of excess heme. Polymerisation of heme into an insoluble, nontoxic crystalline compound, hemozoin (also called malaria pigment) is believed to be prominent way of detoxification. Therefore, the inhibition of hemozoin formation is an attractive target towards development of antimalarial drugs such as 4-aminoquinolines and also considered as suitable target for drug screening methods. Various *in vitro* assays based on spectral characteristics and different solubility of monomeric heme and β-hematin (synthetic analogue of hemozoin) have been used for screening of natural antimalarial as well as novel synthetic compounds.

In the present study the percentage yield of all the extracts was calculated and showed higher yield with ethyl acetate extract followed by chloroform extract of CG flower. The presence of various phytoconstituents were more in ethyl acetate extract of CG flower especially sterols which is responsible for antimalarial activity. An earlier report also revealed the same where ethyl acetate extract showed the presence of sterols and flavonoids (Younes *et al*., 2018). Thereafter, dose dependent antimalarial activity showed with the ethyl acetate and chloroform extracts which were also correlated with the earlier report (Nardos and Makonnen, 2017; Herraiz *et al*., 2019). In the literature, it was revealed that due to the presence of sterols, alkaloids and flavonoids in the plant body, they show antimalarial activity (Kohler *et al*., 2002; Chander *et al*., 2016; Meesala *et al*., 2017; Laryea and Borquaye, 2019) which was similar with our findings where ethyl acetate flower extract showed the presence of sterols, especially Stigmasterol and confirmed with the antimalarial activity.

5. Conclusion

Among various extracts of CG flowers, ethyl acetate extract showed the potent anti-malarial activity in β-hematin formation assay. Further isolation of the compound that is responsible for the activity was carried out and characterized to determine the structure of the compound and the compound was found to be Stigmasterol.

Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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