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BIOASSAY GUIDED FRACTIONATION AND IDENTIFICATION OF BOUNDARY FRACTIONS OF *Arnebia nobilis* : SEARCH FOR ANTIMALARIAL POTENCY IN VITRO

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Abstract

Background:- In today's life, too many diseases are speeding in our surrounding environment. In this research an alternative type of drug is synthesised that is obtained from *Arnebia nobilis* since, Use of same drugs may lead to drug resistance among patients therefore new drugs are needed to be discovered. The species *Arnebia nobilis* being rich source of antimicrobial, anticancer, antiparasitic, febrifuge, and antiseptic and are asserted to be helpful for the treatment of optic, respiratory disease, stomach pain etc. So, we have utilised this plant for antimalarial properties.

Objective:- In this study, assessment of antimalarial potential of *Arnebia nobilis* by in-vitro method is done for being used as traditional medicine against malaria.

Materials and methods:- We subjected the drug to preliminary phytochemical screening, pharmacological screening, physicochemical characterisation (TLC, IR, Mass spectral analysis) involving the antimalarial and cytotoxic assay using Chloroquine diphosphate, Podophyllotoxin as reference drug respectively.

Results:- The results concluded the plant extract exhibits promising antimalarial properties against K1 strain with IC50 value of 7.76 µg/ml (≤10 µg/ml) which indicates 2.3 times less potent than standard drug and that with 3D7 strains IC50 value as 13.73 µg/ml which indicates 2500 times less potent than standard drug. The findings indicate the napthaquinone derivative as the probable reason for antimalarial property.

Keywords: *Arnebia nobilis , Malaria, Napthaquinone derivative, Podophyllotoxin, Antimalarial structures, K1 Strain, IC50.*

Introduction

Natural products and their related products have been wonderfully used as a source of therapeutic agents.[1] It has long been recognized that natural product structures are characterized by large chemical variations, biochemical specifications and other molecular properties that make them attractive as lead structures for drug discovery and serve to distinguish between synthetic and synthetic libraries. [2]

The genus Arnebia belongs to the family of Boraginaceae which is a rich source of alkannin and the shikonnin class. *Arnebia nobilis* is a commercially available species known as "Ratanjot", one of the main sources of arnebin quinine.[1] It can be a potent therapeutic agent for wound healing in steroid-treated wounds. Hydroxynaphthoquinones and particularly isohexenylnaphthazarines which are found naturally are called shikonin and alkannin. They are are lipophilic red pigs which can be taken out by roots of many species with Boraginaceae family. These have shown good antineoplastic, wound healing and action against microbial infection. The roots have febrifuge effect, antihelmenthic and antiseptic potency and are asserted to be helpful for the treatment of optic, respiratory disease, stomach pain etc.[2] Naphthaquinones (arnebins), active compounds of the plant are responsible for their color and therapeutic effectiveness.

The chief motive of this work is to distinguish and clarify the core structure from the bark removal.

MALARIA is usual disease among diseases borne out of insects. Each year around two million people die, and infected people ranges from an estimated 300-500 million people. Approximately there is risk to half of the world's population by fatalities rising substantially among young children under five. Malaria is a disease affecting the capacity of people their families and at last the whole community. Malaria usually occurs in poor and underdeveloped regions. Africa is facing its biggest clash. [3] China, East Asia and India are other hard-hit tropical parts. Specialists estimate 40% malaria of India cases are due to Plasmodium falciparum. [4] The main tree for antimalarial action was found to be quinine, separated by Cinchona bark species (Rubiaceae) within the 1820s. It is one among the oldest and most vital drugs, still in use today. In 1940, another chloroquine was produced for the antimalarial potential, it is well known today for treating malaria. Regrettably being successful in initial stage, the malarial parasites and particularly P. falciparum did not resist chloroquine. The treatment of chloroquine-treated malaria was wiped out alternative ways of shooting up or combinations of medicine, which were expensive and sometimes toxic.

Malaria is violent and destructive, and has in the past been well distributed over humid and arid lands, coastal and forested, overcity scapes and rural landscapes, insubarctic, temperate, tropical, and tropical. Throughout the twentieth century, malaria lost its grip on the northern hemisphere, and in those liberated countries traditional knowledge of malaria was depleted. Today, malaria is a disease that has been virtually forgotten in many Western lands.

Important features of Malaria:

Mosquitoes have tendency to move to feed at the time when humans are sleeping..

- Hosts for plasmodiaum are both humans and mosquitoes.
- After infection caused by malaria, there are visible symptoms promptly, this can take time of nine days to one month.
- Patterns of rain in local area, site where mosquito breeds and variety of mosquito species present are the key factors in transmission. These factors are common in many regions, the whole year and some have specific season in spreading of malaria.

The genus Plasmodium is specific for malaria but four main species are responsible for it, out of four, prominent is Plasmodium falciparum for causing malaria. Main area for spread of malaria are home where the most deadly mosquito Anopheles whose female are responsible in spreading of malaria as described in Figure-1.[7]

The malarial spread-

In 2010 there were 99 countries and areas with persistent malaria transmission and 7 countries in

the ban on recycling, making 106 countries where malaria is considered to be the only disease. In July 2011, South Sudan became an independent country, increasing the number of countries and territories through the ongoing transfer of 100 countries and total destinations to 107. In October 2011, Armenia was confirmed free of malaria by the WHO, reducing the number of malaria countries to 106 countries. As 2010 is the latest year for most information, South Sudan and Sudan results are reported from the same country. However in the country profiles and plug-ins, data from the top transfers and sub-depots are reported separately.

Various researchers are studying and thinking of developing new functional derivatives that can act as substitute to chloroquine and artemisinin which are basically plant based derivatives to be used as drug like artemisinin being obtained from the Chinese plant Artemisia annua. This results to conclusion that plants can be the origin of novel drugs against malaria by looking at favourable action of two agents used as chemotherapeutic like quinine and artemisinin as they are obtained from plants [6].

Our aim is to evaluate the potential of in vitro antimalarial potency extracts of *Arnebia nobilis* and subsequent bioassay isolation to determine the effective action potential lead.

Material and Methods

Plant Material:

Bark specimen was taken from Khari Baoli Street, Chandni Chowk, New Delhi and Dr (Mrs) Sunita Garg, Senior Expert at Raw Material Herbarium and Museum, Delhi (RHMD), CSIR-NISCAIR carried out validation. The discount was given on voucher coded NISCAIR / RHMD / Consult / 2016/2969/162 was subsequently submitted to NISCAIR by the RHMD department.

Preliminary phytochemical screening :

Parts of the plant were dried and de- stroyed as powders, grinders and fine powder were obtained by siege. One gram of powdered root was dried distinctly by taking 10ml solvents such as DMSO, Ethanol, Petroleum Ether, Chloroform, Acetone, Methanol, DCM, Ethyl acetate, Water, and n-Hexane, Methanol-Water (70:30), butanol-Acetic acid-Water; 4: 1: 5 and Ethyl Methyl Ketone in separate test tubes. The powdered drug mixture and solvent were sealed using cotton plug which was then let 48h to stand. After the said time cotton plug removal was done and content was filtered. Testing was made on the filterate by suitable assays [2] for the identification of various phytoconstituents such as flavonoids, alkaloids, glycosides, terpenoids, carbohydrates, proteins and tannins.

Mass Extraction

At room temperature, 250gm of root powdered *Arnebia nobilis* was transported under cold maceration with 400ml ethyl methyl ketone for 7 days. After that it was filtered into a bathtub to obtain a brownish red residue.

Evaluation / pharmacological screening

Fractionation

Combined root extract (40mg) was taken from the china container separately. Silica gel of 200 hp size for column chromatography was taken at a dose of 800mg added with slow pace mixing constantly using metal spatula for achieving good consistency. It was dried by keeping in air and also big pieces were removed for getting good flow mixture.

Column of 5.0 ft. and 16mm height was get hold of and as then dehydrated. The bottom of which was connected by cotton folding. The prepared column was glued, placed vertical at end. Half column filled-up with n-hexane. Small silica gel fractions poured which get settled until it reaches column length. Extract of root which was dissolved in silica was then poured and then sequentially followed by various solutions, respectively n-hexane (10), n-hexane: chloroform (75:25), n-hexane: chloroform (50:50), n-hexane: chloroform (25: 75), chloroform (100), chloroform: toluene (75:25), chloroform: toluene (50:50), chloroform: toluene (25:75), toluene (100), toluene: ethyl acetate (75: 25), toluene: ethyl acetate (50: 50), toluene: ethyl acetate (25: 75), ethyl acetate (100),), ethyl acetate: methanol (75: 25), ethyl acetate : methanol (50: 50), ethyl acetate: methanol (25: 75), methanol (100), methanol: formic acid (50:50), methanol: formic acid: water (4: 1: 5). Conical flasks were taken and marked to collect thirteen fractions which were sent

under TLC for checking their homogeneity. Chromatograph in parallel the various identical fractions (with the same Rf values) were merged and merged.

Physiochemical characterization

Four pure bioactive were separated by column chromatography after performing TLC and the non-specific fraction was correctly identified using appropriate analysis. TLC, I.R & Mass Spectroscopy were done for other identification and calibration.

- Review of TLC

Ethyl acetate: n-hexane (7: 3) was used initially for TLC analysis for different constituents from plants, and separately single constituent was checked by specific TLC analyzes using specific Toulene systems : Form Acid (8.5: 1.5) as the cellular component of each class of phytoconstituents, already known for the analysis of the relevant compounds.

- Infra red (IR) Spectral analysis

SHIMADZU FTIR-AFFINITY 1 was used to perform IR spectra having range from 4000 to 400 cm-1 in the form of a KBr disc. Individual constituent to be given I.R by a rigorous potassium bromide-pelleting process by studying IR, the functional groups are clearly identified and are a powerful tool for the oxidation process. 200 mg of KBr of each pellet was dried in an oven at 110°C. The 2mg root extract was mixed with KBr and the pellet was prepared using a piercing machine using a pressure of 8-10 tons. Pellet was placed on the disc and its absorption was taken.The visual spectrum was obtained.

- Mass Spectral Analysis

Thermo Finnigan LCQ Advantage max ion trap mass spectrometer at Jubiliant Chemsys, Greater Noida was used to get the electrospray signal of mass recorded for all the compounds. 10µl test compounds (dissolved using methanol / DMSO) imported in ESI source which was done by using Finnigan Surveyor auto-sampler. The mobile phase (90:10 MeoH / ACN: H2O) flowed at a rate of 250µl / min by Ms pump. Ion spray voltage is set to 5.3 KV and capillary voltage 34V. The MS scan reaches 2.5 meters and the spectra print is located above the 10 scan above the TLC. The mass spectra provide information on the different types of peaks and determine the method of collection of single cells after successful interpretation. [6]

Acute toxicity studies

An acute toxicity test can provide initial information on the toxicity of something not found in other toxology data. The animals were processed and all criteria were approved by the CPCSEA (No. IEC / KSOP / E / 15/001).

Description of the method

- Species selection: Feeding Women taken (not pregnant). At start of dosage 8-12 weeks animals used
- Living and feeding conditions: 22°C + 3°C.
- Preparation of doses: 1mL / 100g of body weight and not to exceed this volume.

Procedure

1. The test material is delivered by single dose with gavages which was done by stomach tube or ventilator. When in some cases there was not possible to give single dose, small fractions were given over a period of not more than 24 hr.

2. Animal feeding is to be done prior to dosing like when rat is food given but no water overnight and if mouse then food given but water is not given for 3-4 h. After this rats and mouse are weighed and the test compounds under control. After packing of item, the food may be withdrawn from animals for additional 3-4 hr in the case of mice. Where fractions are taken over a period of time it is not necessary to provide animals with food and water depending on the length of time.

Number of animals and dose levels

1. Three animals are used for each step. The dose to be used as the first dose is selected from one of the four solid doses of 300 mg / kg body weight.

2. Initial dose taken would produce death mostly in some restricted species. Flow Chart Annexure 2 Figure 2 describes the procedure to be followed for each implementation volume.

3. Where the available information suggests that death cannot be reached at the dose of 2000 mg / kg body weight which is maximum level of dose

given so limit assessment can be done for this. There are recommendations for using 300 mg / kg as initial dose for test samples where limited information is given about test congeners for welfare of animals.

4. Initiation, duration and severity of side effects determines the time of differentiation between treatment classes. After convincing that he or she will be able to survive than the previously released animals, experiment for next treatment should be done.

5. Except, and only where appropriate for specific regulatory requirements, maximum dosage to be considered is 5000 mg / kg of body weight. Considering animal welfare, there is discourage of testing at GHS Category 5 ranges (2000-5000mg / kg) and so is carried out only when more chances of result being strongly correlated with protection of animal or individual health.

Bio evaluation of phyto compounds for antimalarial activity

Experimental model: - In vitro - Plasmodium falciparum (3D7 and K1 Strains)

In vitro cultures of both Chloroquine - sensitive (3D7) and (resistance K1) of Pen falciparum were maintained in RPNI supplemented with 25 mM HEPES, 0.2% D-glucose, 0.21% sodium bicarbonate and -0.5% ALBUMAX-II. . [4] The stock solution (10 mM) of the computer was prepared in DMSO and the necessary dilutions were prepared in medium culture. Analysis of 50% Inhibitory concentration (IC50) of the compound, Malaria SYBR Green Based fluorescence (MSF) assay (Singh et al, 2011) was performed.

Assay for Antimalarial Activity

The maximum amount of the test sample was 50.0 µg / ml. Sequential distribution were made from 96-well plates and were subjected to a 1.0% cell suspension containing 0.8% parasitaemia (Asynchronous culture with a maximum of 80%). The plate was incubated at 37oC in a CO_2 incubator with 5% CO_2 and air mixture. After a time of 72h, buffer of lysis of amount 100 µl containing 2x concentration of SYBR Green-I (Invitrogen) was allowed to include in all the wells which were then for 1 hour at 37oC incubated. The plate was monitored at 485 ± 20nm of emission and 530 ± 20nm emission fluorescence units (RFUs) using a well-established fluorescence plate (FLX800, BIOTEK). Logit regression analysis was done to determine the IC50 of the reaction-reaction curves. The reference taken in this experiment was chloroquine diphosphate (SIGMA).

Formula used for selecting lead:

IC50 = ≤10.0 µg / ml

Cytotoxicity assay

The assay for checking the cytotoxicity of the samples was done on Vero cell line (C1008; Monkey fig fibroblast cell) using instruction of the method as described [5]. Cells were subjected to a 72hr sample dilution and MTT was used as a reagent for cytotoxicity. The maximum amount of the sample taken was 100µg / ml. 50% cytotoxic concentration (CC50) which was determined using flow-reaction curves. The reference substance here used is Podophyllotoxin (SIGMA).

The formula used to determine selectivity index (SI) is as:

SI = CC50 / IC50

Results

- Preliminary Screening Phytochemically

Examination results of phytochemical test of the roots of *Arnebia nobilis* represented in Table-1.

The results clearly showed that ethyl- methyl ketone contains high active sites.

- Mass Extraction

The average yield of filtered residues after the cold discrimination was 31.51gm and the percent yield was 40%.

- Acute toxicity studies (Anti-Malarial)

Observations

1. Animals kept separately when being drawn for once for the first 30 minutes, occasionally for the duration of first 24 hours, attention should also be given for initial 4hrs and then daily ofr next 14 days except when required to be removed from research and killed by humans for animal welfare reasons or found dead. The times when signs of poisoning are

visible and go are significant mainly when delaying of symptoms may occur.

2. If animals show continuous toxicity reactions, additional surveillance would be required. Recognition should be done of eyes, skin related, body tissues, patterns of behaviour, circulatory system, somatomotor and central nervous system. Focus should be mainly on shaking, jumping, threatening, dragging, killing, sleeping and pain.

3. Weighing of the animal must be done just prior to giving test component and then atleast weekly. Calculations and recording of change in weight should be done and after experiment ends animals are again weighed and killed.

- Primary Identification and Isolation of Bioactive leads of *Arnebia nobilis*

Four different fractions were obtained after crossing the column with *Arnebia nobilis* root extract, 4 (n-hexane-chloroform) (25:75) - [F4], part 11 (Toluene-Ethyl acetate) (50:50) - [F11], part 3 (Toluene-Ethyl acetate) (25:75) - [F3] and 4 (Ethyl Acetate-Methanol) (75:25) - [F4] .The fractions were examined for initial phytochemical testing and the results were is given in Table-2.

- Physico chemical characterization of bioactive lead from *Arnebia nobilis*

Structure characterization done by Mass spectroscopy of single bioactive leads, F1, F2, F3 and F4. Primary phytochemical examination and test results of TLC show that the pure compound was present and is represented in table 3,4,5,6 and then calculated structure in figure 3,4,5,6.

- Final structure of the compound F1 (Figure 3)

Arnebin-1

Mol. Weight : 370

Molecular formula : C21H22O6

Napthaquinone derivative

- Final structure of compound F2(Figure 4)

Isorhamnetin

Mol. Weight : 316

Mol. Formula: C16H12O7 (Glycoside)

- Final structure of the compound F3 (Figure 5)

Diosmetin (Flavanoid)

Mol. Weight :C16H12O6

Mol. Weight : 300

- Final Structures of compound F4(Figure 6)

Lindechunin A (Isoquinoline alkaloid)

Mol.weight: 351

Mol. Structure:C19H13NO6

❖ Spectral Datas-
The spectral datas of all the compound are shown in Figure 7,8,9,10 respectively.

- Anti Malarial and Cytotoxicity Assay

The values of IC50 and CC50 for the test sample are shown in Table-5. It is observed that the extraction of the plant showed promising antimalarial activity against the K1 strain while in contrast to the complexity of 3D7 activity was limited.

It is suggested that studies can be further developed for the addition of fractions.

Table 7 shows In vitro Antimalarial and Cytotoxic activity.

Discussion

Ethyl methyl ketone extract from roots of *Arnebia nobilis* derived from in-vitro cultures of both chloroquine (3D7) and potent (K1) plasmodium falciparum clearly showed that the above extracts were found to have higher anti-K1 strains in the amount of IC50 as 7.76 µg / ml and low anti-malarial potency against 3D7 IC50 values as 13.73 µg / ml respectively compared with standard QC-diphosphate with IC50-related values as 0.005 µg / ml with -0.338 µg / ml against 3D7 and K1 strains. These results clearly showed that the test output was 2.3 m stronger than normal (strain K1) and 2500 times lower than normal (3D7 layers). The analysis makes the prediction that our extracts are more effective against K1 strains, while less effective against 3D7 strains, in such a way that our drug promises to lead against K1 strains as IC50≤ 10µg / ml.

The extraction of the plant was also considered to be divided into four functional zones i.e. Lindechunine A, Diosmetin, Isorhamnetin and Arnebin-1 for structural reorganization by Mass Spectral analysis. Previous literature describes the Arnebin-1 functional domain responsible for the therapeutic efficacy of the compound. In our research, we have identified four different classes of active sites namely isoquinoline alkaloid (Lindechunine A), Flavonoid (Diosmetin), Glycoside (Isorhamnetin) and napthaquinone derivative (Arnebin-1), which may be a possible reason for the high magnitude of resistance to the K1 subclasses falciparum. This is the first investigative report, that three new classes of compact Lindechunine A, Diosmetin, and Isorhamnetin are present in the plant pump, which may account for the selectivity of the K1 strain.

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Table 1. Results of preliminary phytochemical screening.

	ALKALOIDS		GLYCOSIDES				FLAVONOIDS		VITAMINS		
	Dragendroff test	Hagers test	Borntragers test	Mod.borntragers test	Legal test	Baljet test	Ammonia test	Sulphuric acid test	Vitamin A test	Vitamin D test	Vitamin C test
DMSO	-	++	++	++	-	-	-	+	-	-	-
n-hexane	++	+	-	+	-	+	+	++	-	-	+
Pet.ether	+	+	-	+	-	+	++	++	-	-	+
Chloroform	+	++	++	-	-	+	+	-	-	-	+
Dichloromethane	++	-	-	++	-	++	+	-	-	-	-
Acetone	-	-	++	-	-	++	-	-	-	-	-
Ethyl acetate	++	+	++	+	-	+	+	+	-	-	-
Methanol	+	++	++	-	+	+	++	++	-	-	-
Ethanol	++	-	++	+	-	+	-	-	-	-	-
Water	+	++	-	-	-	+	+	+	-	-	+
Methanol+water	-	+	+	-	-	-	-	+	-	-	+
BAW	++	-	+	+	-	+	-	-	-	-	-
Ethyl methyl ketone	-	++	++	+	+	++	++	++	-	-	-

Table 2. Screening Results

SCREENING RESULT FOR ISOLATED FRACTIONS

	ALKALOIDS		GLYCOSIDES				FLAVONOIDS		VITAMINS		
	Dragendroff test	Hagers test	Borntragers test	Mod.borntragers test	Legal test	Baljet test	Ammonia test	Sulphuric acid test	Vitamin A test	Vitamin D test	Vitamin C test
FRACTION 1	++	+	+	++	-	+	-	-	-	-	-
FRACTION 2	++	++	+	+	-	+	-	+	-	-	-
FRACTION 3	++	+	-	++	-	++	-	+	-	-	-
FRACTION 4	++	++	-	++	-	+	+	++	-	-	-

Table 3. Fraction 1 (F4) of *Arnebia nobilis* c

Mass	Ion	Product ion and composition of the neutral particle lost	Corresponding peak with m/z value	Substructure or compound type
1	-	$[M+1]^+$	371	Molecular ion with protonated ion peak
17	OH^+	$[M-17]^+$ (OH)	353	N - oxides
19	H_3O^+	$[M-19]^+$	351	Abundant ion in M-19 (base peak)
67	$C_5H_7^+$,$C_4H_3o^+$	$[M-67]^+$	303	Furyl Ketones
68	$C_5H_8^+$, $C_4H_4O^+$	$[M-68]^+$ (C_5H_8) (C_4H_4o)	302	Cyclohexenes Cyclohexenones
87	$C_5H_{11}o^+$	$[M-87]^+$	283	Alcohols,ethers,esters 'O' indicators
88	$C_4H_8O_2^+$	$[M-88]^+$	282	α – methyl- methyl esters
131	$C_{10}H_{11}^+$	$[M-131]^+$	239	Tetralins

Table 4. Fraction 2 (F11) of *Arnebia nobilis*

Mass	Ion	Product ion and composition of the neutral particle lost	Corresponding peak with m/z value	Substructure or compound type
-	M^+ molecular ion	-	316	Molecular ion of glycoside type
34	$(OH)_2^+$	$[M-34]^+$ (OH+OH)	282	-
35	-	$[M-35]^+$ (OH+H_2O)	281	2× 'O' Indicator as base peak
36	C_3^+	$[M-36]^+$ (H_2O+H_2O)	280	2× 'O' Indicator
83	$C_6H_{11}^+$ $C_5H_7O^+$	$[M-83]^+$ $[M-83]^+$	233	Alkenes, Alicyclics, Cycloalkanones.

Table 5. Fraction 3 (F12 &F13) of *Arnebia nobilis* c

Mass	Ion	Product ion and composition of the neutral particle lost	Corresponding peak with m/z value	Substructure or compound type
-	M^+	-	300	Molecular ion of flavonoid type
21	$C_2H_2O^{++}$	$[M-21]^+$	279	-
43	$C_3H_7^+$ $C_2H_3O^+$ CH_3CO^+	$[M-43]^+$ (C_3H_7) $[M-43]^+$ (CH_3CO)	257	Alicyclics, cycloalkanones,cycloalkanols, Aromatic methyl ethers.
44	$C_2H_4O^+$ CO_2^+	$[M-44]^+$ (C_2H_4O) $[M-44]^+$ (CO_2)	256	Cycloalkanols, cyclic ethers, Lactones.
88	$C_4H_8O_2^+$	$[M-88]^+$	212	α- methyl-methyl esters
105	$C_8H_9^+$ $C_7H_5O^+$	$[M-105]^+$	195	Alkyl aromatics Benzoyl derivative.

Table 6. Fraction 4 (F14) of *Arnebia nobilis*

Mass	Ion	Product ion and composition of the neutral particle lost	Corresponding peak with m/z value	Substructure or compound type
1	-	$[M-1]^-$	350	Occurs for moderately basic & acidic compound
31	CH_3O^+ 'O' Indicator	$[M-31]^+$ (CH_3O)	320	Methyl ethers and primary alcohols.
50	$C_4H_2^+$	$[M-50]^+$	301	Aromatics
72	$C_4H_8O^+$ $C_4H_{19}N^+$	$[M-72]^+$	279	Alkanones- 'O' Indicator Alkenes- 'N' Indicator

Table 7. In vitro Antimalarial and Cytotoxic activity

S.no.	Sample code	IC_{50} (µg/ml)		CC_{50} (µg/ml)
		3D7	K1	
1	Plant extract	13.73	7.76	>100
QC- diphosphate*		0.005	0.338	175
Podophyllotoxin*		NA	NA	5.4

* Values in µM

Figure 1. Plasmodium Reproductive cycle

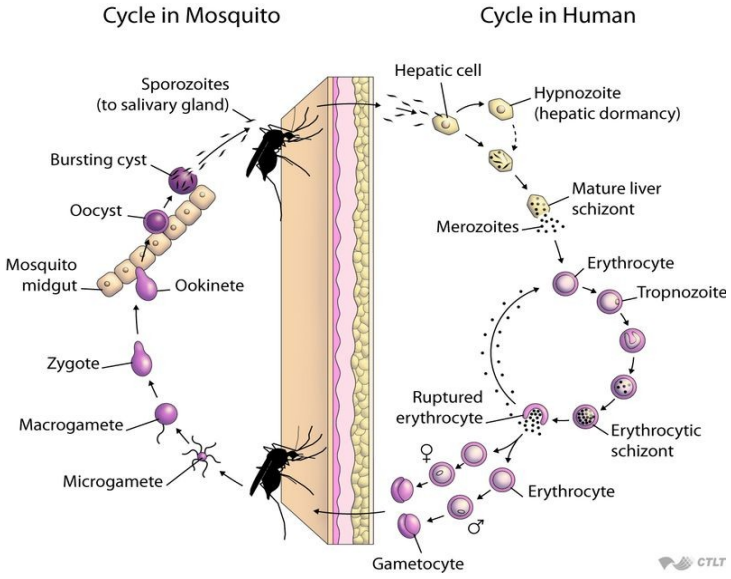


Figure 2. ANNEX 2C: Test procedure with a starting dose of 300 mg/kg body weight.

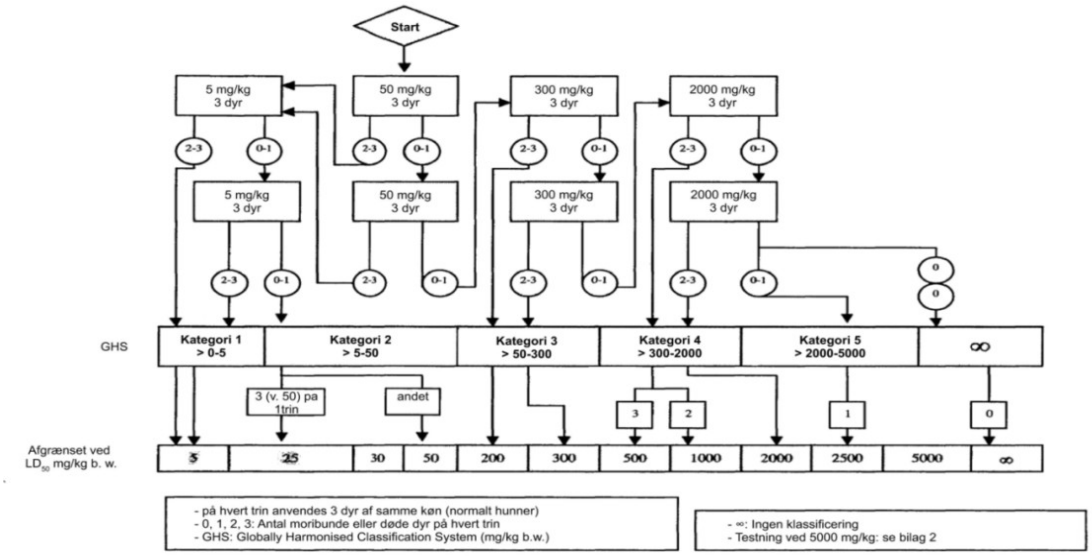


Figure 3. Arnebin-1

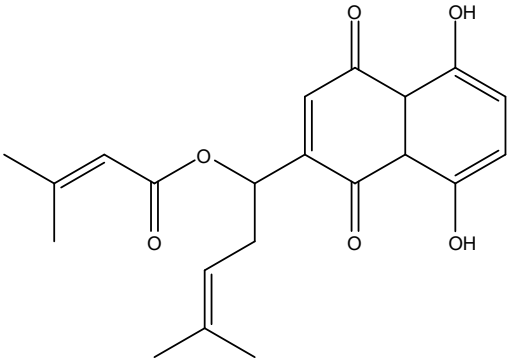


Figure 4. Isorhamnetin

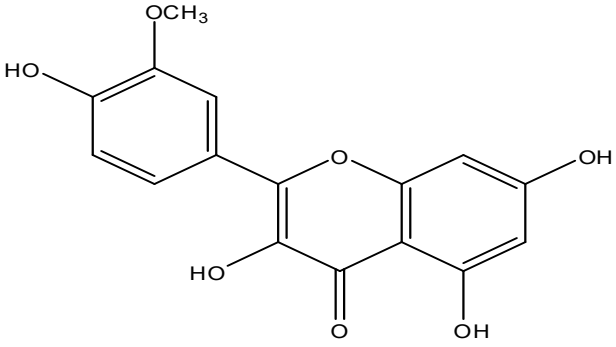


Figure 5. Diosmetin

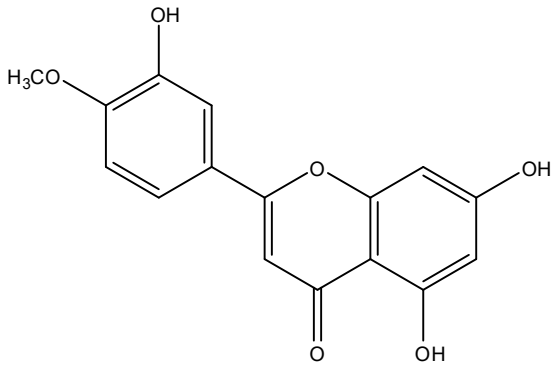


Figure 6. Lindechunine A

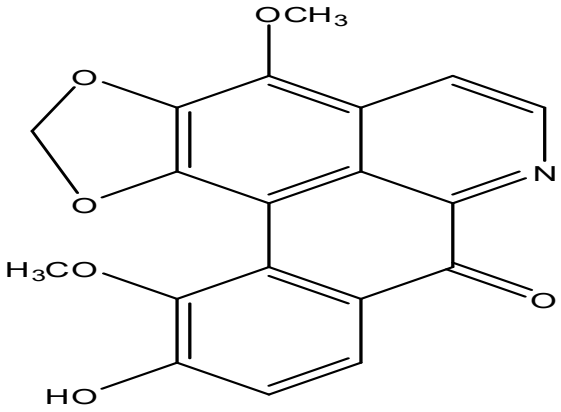
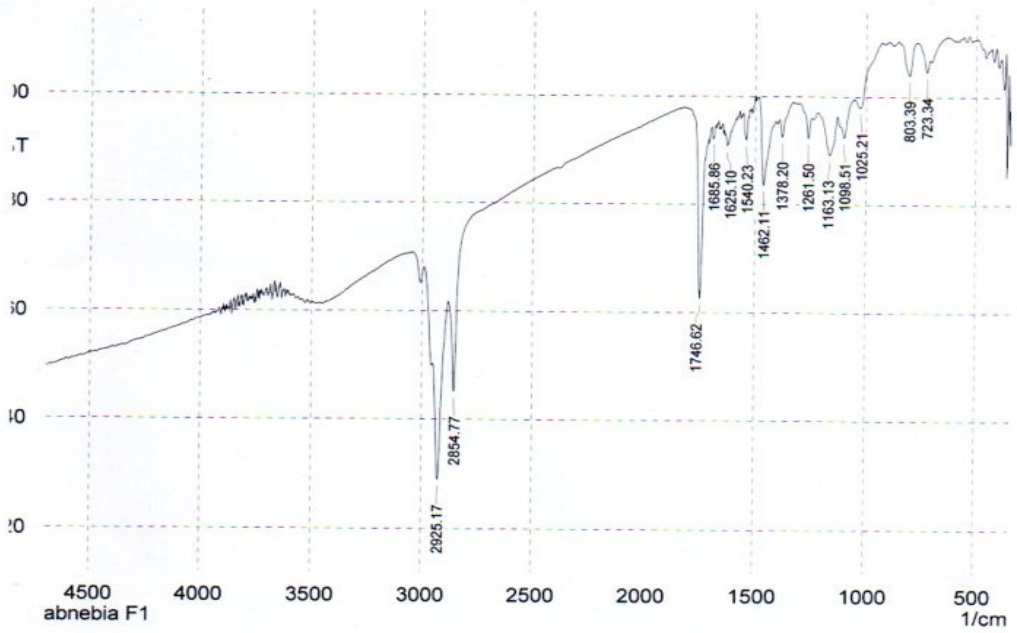
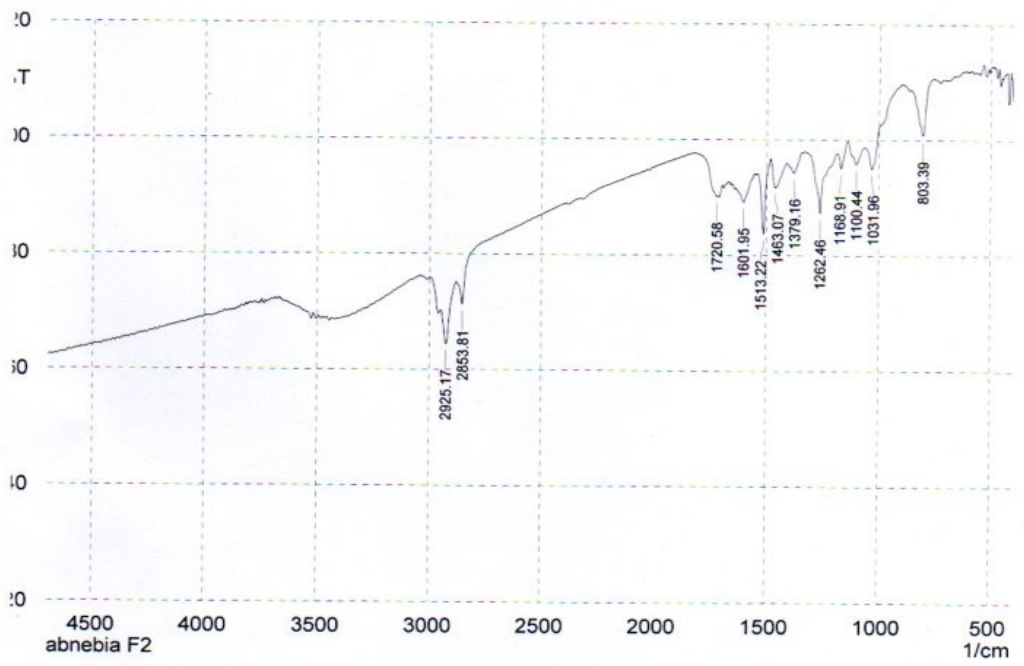


Figure 7. Spectral data of Fraction F1



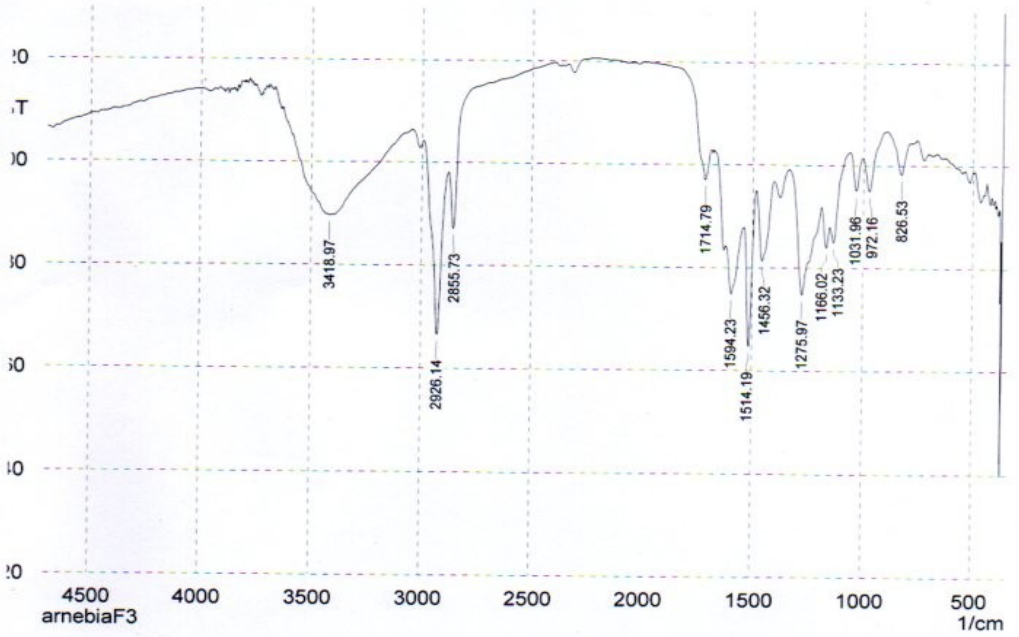
o.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
	723.34	104.563925	3.058644	769.63	708.87	-1.935453	0.205605
	803.39	103.960821	6.163448	854.5	769.63	-2.736234	0.82309
	1025.21	98.037889	3.614835	1050.29	926.84	-2.165828	0.259249
	1098.51	92.413122	3.308709	1113.94	1050.29	1.056967	0.210231
	1163.13	89.171971	7.471462	1218.1	1129.37	2.73629	1.456931
	1261.5	92.310623	4.471853	1298.15	1247.03	0.912456	0.319727
	1378.2	92.522511	0.144641	1379.16	1329.98	0.732169	-0.202398
	1462.11	83.537181	14.502174	1483.32	1420.63	2.747858	1.953586
	1540.23	91.941621	4.674301	1552.76	1530.58	0.601142	0.272796
	1625.1	90.843051	2.420036	1633.78	1608.7	0.901402	0.15164
	1685.86	92.304377	0.531133	1687.79	1676.21	0.341575	0.010085
	1746.62	62.588289	29.43057	1772.66	1725.4	4.815392	3.177495
	2854.77	45.161113	17.987832	2881.77	2390.87	51.812307	-14.86358
	2925.17	28.998135	25.496674	2950.25	2881.77	24.259114	6.876511

Figure 8. Spectral data of Fraction F2



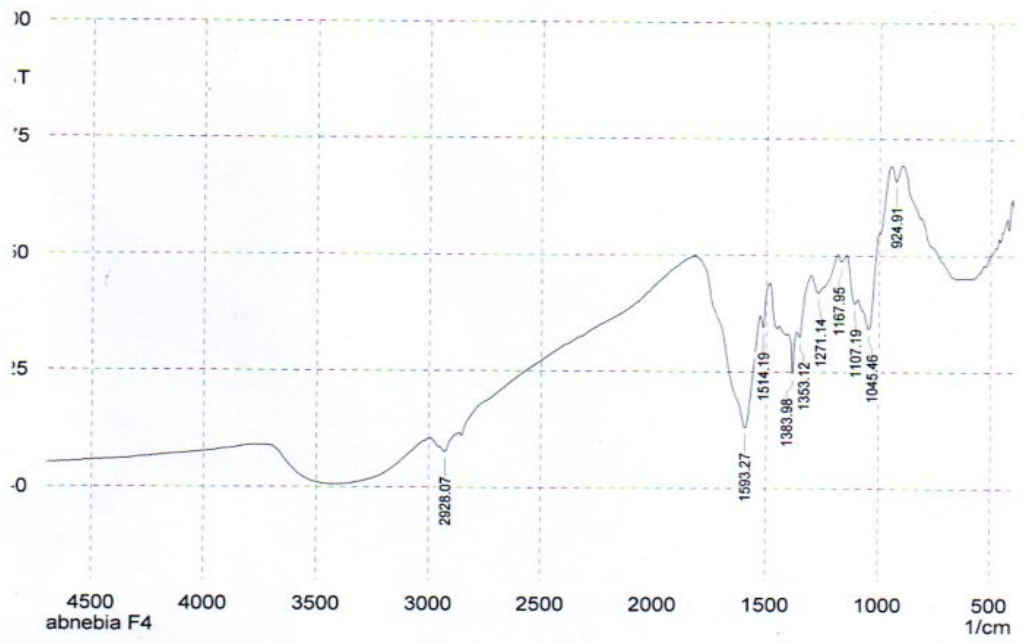
o.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
	803.39	100.8314	8.902	857.4	758.06	-2.5989	1.3722
	1031.96	94.9766	5.7538	1062.82	894.04	-2.2298	0.6778
	1100.44	95.8436	3.4057	1139.98	1062.82	0.8358	0.5873
	1168.91	95.0622	3.343	1185.31	1139.98	0.5631	0.3053
	1262.46	87.2998	10.4989	1331.9	1185.31	3.5154	2.0924
	1379.16	94.255	2.5681	1410.99	1343.48	1.3168	0.3852
	1463.07	91.6799	0.8029	1482.36	1460.18	0.6145	0.0455
	1513.22	83.6671	10.2488	1540.23	1499.72	1.9137	0.8168
	1601.95	89.1837	2.2744	1618.35	1566.27	2.2132	0.3061
	1720.58	90.1389	0.1971	1818.95	1718.65	2.3065	-0.4537
	2853.81	71.2662	4.8852	2880.81	2645.48	22.1138	-1.3357
	2925.17	64.4067	7.5416	2949.29	2880.81	10.7816	1.2715

Figure 9. Spectral data of Fraction F3



o.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
	826.53	98.2673	6.9258	893.08	788.92	-1.2304	1.1944
	972.16	95.0368	7.8814	1003.99	893.08	-0.7246	1.1666
	1031.96	95.1212	6.8688	1059.93	1003.99	0.2765	0.7548
	1133.23	84.9812	5.8423	1150.59	1059.93	2.1065	0.0986
	1166.02	84.023	5.63	1188.2	1150.59	2.2792	0.5552
	1275.97	74.8132	21.4253	1336.73	1188.2	9.8408	6.8799
	1456.32	81.3684	14.297	1484.29	1405.2	4.3861	2.9572
	1514.19	64.6862	26.4893	1539.26	1484.29	5.6087	3.4664
	1594.23	74.8822	10.6172	1620.27	1543.12	7.2451	2.2034
	1714.79	97.0659	8.6702	1830.52	1689.72	-5.1371	1.0026
	2855.73	87.1874	13.0817	2878.88	2694.67	-6.6441	-1.6161
	2926.14	66.5189	34.0929	2993.65	2878.88	7.1935	7.7443
	3418.97	89.6763	0.1769	3428.62	3411.26	0.8156	0.0098

Figure 10. Spectral data of Fraction F4



o.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
	924.91	65.9807	3.2674	946.12	895.97	8.4752	0.4763
	1045.46	34.2525	13.5324	1092.72	999.17	36.7212	6.1919
	1107.19	39.6719	3.5039	1145.77	1092.72	19.3103	0.972
	1167.95	48.6997	1.62	1182.41	1145.77	11.218	0.2799
	1271.14	41.912	5.0779	1303.94	1182.41	42.3665	3.6405
	1353.12	32.5505	3.625	1366.62	1303.94	25.7706	0.2536
	1383.98	24.7354	8.6382	1405.2	1366.62	19.9649	1.5684
	1514.19	34.5951	4.7401	1526.72	1489.11	15.4723	0.6338
	1593.27	13.0071	26.9848	1818.95	1526.72	156.2847	49.1556
	2928.07	7.6171	3.3988	2992.69	2865.38	130.2057	8.1288