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Isolation and pharmacological activities of hypericin fraction from the leaves of *Hypericum hookerianum* Wight & Arnott

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Abstract
Hypericum hookerianum commonly known as Hooker's St. John's Wort belongs to the family Hypericaceae. It helps in treatment of chronic diseases, especially schizophrenia. The study has been done on the methanol leaf extract of the plant. It aimed at evaluating the antioxidant and antibacterial activities of hypericin fraction. The maximum 1, 1- diphenyl 2-picrylhydrazyl (DPPH) radical scavenging activity of methanol extract of leaves of hypericin fraction was 59.36 ± 0.49% at 120 µg/mL concentration and the IC_{50} was 79.05 µg/mL concentration. The maximum superoxide radical scavenging activity of hypericin extract was 36.05 ± 0.09% at 120 µg/mL concentration and the 50% inhibitory concentration (IC_{50}) was 166.41 µg/mL concentration. The maximum Fe^{3+} reduction was 84.81 ± 0.12% with the 50% reduction concentration (RC_{50}) of 20.05 µg/mL and the maximum Mo^{6+} reduction was 99.25 ± 0.35% at 120 µg/mL concentration with the RC_{50} of 10.14 µg/mL concentration.

Keywords: *Hypericum hookerianum*, DPPH, Hypericin fraction, GC-MS

1. Introduction
Hypericum hookerianum is a perennial shrub is native to Southern and Eastern Asia. In India, it is found in the higher altitudes of Nilgiris, Khasi and Jaintia hills of Himalayas and Palani hills of Western Ghats. *Hypericum hookerianum* ranges from 6–8 ft (1.8–2.4 m) in height [1]. The terete branches of the shrub are reddish brown in colour. The leaves belong to the largest genus, measuring between 2–4 inches (51–102 mm) long. The leaves extract of the plant is used to enhance the spontaneous motor activity of the body. *Hypericum hookerianum* can be found in southern India, the Himalayas, from north-western Thailand to Bangladesh, China and California [2, 3]. *Hypericum hookerianum* is used as an herbal medicine for HIV treatment and schizophrenia. It can act as an antidepressant and ant proliferative. It has antibacterial activity and anti-inflammatory activities. The leaves extract of *Hypericum hookerianum* has strong antioxidant property [4]. Antioxidants are substances that inhibit oxidation which helps in preventing excess production of free radicals. Hyperforin is also responsible for inducing the P450 liver enzymes as it increases the metabolism of other drugs in the body. The principle reason behind the use of hypericum in traditional medicine is they accumulate pharmacologically important therapeutic compounds such as hypericins and hyperforins. Hypericin and hyperforin both can act as potent photodynamic therapy against cancer.

2. Materials and methods
2.1 Collection of plant material
Hypericum hookerianum leaves were collected from Pine forest, Kodaikanal, Tamil Nadu, India. The collected leaves were shade dried and stored in a paper cover at room temperature.

2.2 Preparation of extract by Soxhlet method
The shade dried leaves of *Hypericum hookerianum* was crushed, using mechanical blender and about 20 g of coarse powder was loaded and packed in a (12× 3 cm) thimble made from a strong filter paper which was palced inside the thimble chamber of soxhlet extractor. The solvent 250 mL of methanol was added to a round bottom flask, which was attached to a Soxhlet extractor and condenser. The whole set up placed onto the mantle. The side arm was lagged with cotton. The solvent was heated using the heating mantle at 70 ˚C and will begin to evaporarte, moving through the apparatus to the condenser. The condensate then drips back into the thimble container. Once the level of the solvent reaches the siphon arm, the liquid contents emptied into the round bottom flask and the cycle begins again [5].

The process is run until the thimble turns colourless. The supernatant in the round bottom flask was condensed in a rotary evaporator at 50 ˚C to yield the extract. Then the hypericin fraction was eluted by column chromatography using CH_3OH as eluting solvent.

2.2 Antioxidant assays

2.2.1 DPPH˙ radical scavenging activity

Based on the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical, the antioxidant activity of hypericin fraction was measured [6]. 1 mL of different concentrations (20-120 µg/mL) of hypericin fraction was mixed with 1 mL of 0.1 mM DPPH solution in methanol. The mixture was incubated in the dark for 30 min. The reduction in absorbance was measured using UV-Vis Spectrophotometer at 517 nm (Systronics, Spectrophotometer 104). Ascorbic acid was used as the positive control.

The percentage of inhibition was carried out using the equation

$$\%\ of\ inhibition = \frac{control - sample}{sample} \times 100$$

2.2.2 Superoxide radical scavenging activity

By following the method of Ravishankara *et al*, [7] superoxide radical scavenging activity was carried out. The reaction mixture with different concentrations of hypericin fraction (20 - 120 µg/mL), 200 µL of 1.5 mM of riboflavin, 100µL of 12 mM of EDTA and 200 µL of 50 mM of NBT, added into the test tubes sequentially. All the reagents should be prepared in 50 mM of phosphate buffer of pH - 7.6 only. The reaction mixture was illuminated in UV for 15 min. After illumination, UV Vis spectrophotometer (Systronics, Spectrophotometer 104) was used to measure the absorbance at 590 nm and the IC_{50} was calculated. Ascorbic acid was used as the positive control.

The percenttage of inhibition was carried out using the following equation.

$$\%\ of\ inhibition = \frac{control - sample}{sample} \times 100$$

2.2.3 Phosphomolybdenum reducing power assay

The different concentrations ranging from20 to 120 µg/mL of hypericin fraction was combined with phosphomolybdenum reagent solution containing 4mM of ammonium molybdate $[(NH_4)_2MoO_4]$, 28 mM of sodium phosphate $[Na_3Po_4]$ and 600 mM of sulphuric acid $[H_2SO_4]$. The reaction mixture was incubated in water bath at 90 ºC, for 90 min [8]. By using UV-Vis spectrophotometer the absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as the positive control.

The percentage of inhibition was carried out using the following equation.

$$\%\ of\ reduction = \frac{sample - contol}{sample} \times 100$$

2.2.4 Ferric (Fe^{3+}) reducing assay

The method of Jayanthi *et al*., [9] was slightly modified to determine the reduction power of hypericin fraction. 1 mL of hypericin fraction of different concentrations ranging between 20 and 120 µg/mL was mixed with 1 mL of 0.2 M phosphate buffer of pH - 6.6 and 1 mL of 1% (w/v) potassium ferricyanide solution. The reaction mixture was incubated at 50 ºC for 20 min. 1mL of 10% (w/v) trichloroacetic acid (TCA) was added to each mixture followed by 100 µl of 0.1% (w/v) $FeCl_3$ solution and shaken well. The absorbance was measured at 700 nm using UV Vis Spectrophotometer. Ascorbic acid was used as the positive control.

The percentage of inhibition was carried out using the following equation

$$\%\ of\ reduction = \frac{sample - contol}{sample} \times 100$$

2.3 Antibacterial activity

2.3.1 Microbial strains

Gram negative strains such as *Escherichia coli* and *Proteus vulgaris* as well as Gram positive strains such as *Bacillus subtilis* and *Micrococcus luteus* were used for the evaluation of antibacterial activity. Tetracycline possesses a wide range of antimicrobial activity and therefore it was chosen as the standard reference.

2.3.2 Well Diffusion method

According to the standard methods, 100 mL of nutrient broth agar medium was prepared. Agar well diffusion method was done in order to check the antibacterial activity of hypericin fraction. For the inoculation of bacterial culture onto the solidified nutrient agar in the petri plates, sterile L rod was used. Sterile well-borer of 8 mm diameter was used to create five wells in each plate. The hypericin fraction was added to the wells of 250, 375 and 500 µg/mL concentrations. Tetracycline of 25 µg was used as the positive control. The plates with loaded wells were incubated at 37 ºC for a period of 24 hr [10]. The antibacterial activity of hypericin fraction was calculated by measuring the diameter of zone of inhibition that was formed around the well.

2.4 Antihaemolytic assay

Antihaemolytic activity of hypericin fraction was assessed by using human venous blood sample. Human venous sample was collected in EDTA vials from healthy adults. The blood sample was mixed with of 0.9% (NaCl) saline and centrifuged at 1000 *g* for 10 min. The cells that were separated by centrifugation and diluted with 0.2 M of phosphate buffer saline solution of pH - 7.4 (500 µL cells/10 mL PBS). Varying concentrations (20-120 µg/mL), of the hypericin fraction was added in test tubes and make up to 1mL by saline (0.9%). In the reaction mixture 200 µL of blood suspension was added [11]. The reaction mixture was incubated in water bath for 30 min at 50 ºC. In order to obtain 100% haemolysis, 1mL of saline was added to 1mL RBC suspension which was considered to be the control. The decrease in absorbance was measured in UV-Vis Spectrophotometer (Systronics, Spectrophotometer 104) at 540nm.

The percentage of inhibition was carried out using the following equation.

$$\%\ of\ \ inhibition = \frac{(control - sample)}{control} \times 100$$

2.5 Thin Layer Chromatography

Thin layer chromatography (TLC) was used for analysing the components of hypericin fraction of *Hypericum hookerianum* using Merck TLC aluminium sheets, silica gel 60 F254 (20 x

20 cm), preloaded plates. The hypericin fraction was mottled at 0.3 mm above from the bottom of the TLC plate. The spots were visualized in UV light at 254 nm. The retention factor (R_f) value of the coloured spots was recorded and the ratio in which distinct bands appeared was optimized and R_f values were calculated [12].

R_f value was calculated by using the equation

$$Rf\ value = \frac{distance\ travelled\ by\ the\ solute}{distance\ travelled\ by\ the\ solvent}$$

2.6 Angiogenic activity

Seven day-old fertilized eggs were collected from a local hatchery. The eggs were applied with ethanol and incubated at 37 ˚C. A hole was carefully drilled over the egg shell with a nipper so that, the vascular zones were easy to be identified on the Chorioallantoic Membrane (CAM) [13]. 5 mL of albumin was aspirated for loading the disc. The eggs were incubated horizontally in order to allow the CAM to detach from the shell. Discs containing agarose were placed in sterile petri-plates. Hypericin fraction was loaded in agarose discs at concentration of 100 µg/disc. The negative control used here was the Discs containing the vehicle only (DMSO). A small square opening was made in the shell, and the discs were directly applied onto the CAM. The square opening was covered with parafilm tape and was incubated for 48 hr at 37 ℃. The CAMs were viewed and photographed under a dissecting microscope.

2.7 Gas Chromatography–Mass (GC–MS) Spectroscopy

The hypericin fraction sample was loaded into a HP-5 column (30 mm X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model for carrying out GC-MS analysis. Helium was used as a carrier gas of flow rate 1 mL/min, the injector was operated at a temperature of 200 ℃ and column oven temperature was programmed as 50-250 ℃ at a rate of 10 ℃/min injection mode. These were the chromatographic conditions used for carrying out gas chromatography. Similarly, in order to carry out mass spectroscopy, conditions such as, ionization voltage of 70 eV, Ion source temperature and Interface temperature of 250 ℃ with a mass range of 50-600 mass units were maintained [14].

Identification of components

The mass spectrum of GC-MS was analysed with the aid of the database from the National Institute Standard and Technology (NIST). The mass spectrum of the unknown component of *Hypericum hookerianum* sample was compared with that of spectrum of the known components which are stored in the NIST library.

3. Results & Discussion

3.1 Antioxidant assays

3.1.1 DPPH˙ radical scavenging activity

Scavenging of 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical is one of the well-received decolourization antioxidant assays. DPPH radical scavenging assay measures the potency of antioxidants to scavenge DPPH˙ radicals. The absorbance was measured using UV Vis spectrophotometer at wavelength of 517 nm. The maximum DPPH˙ radical scavenging activity of hypericin fraction was 59.36±0.49% at 120 µg/mL concentration (Fig. 1),(Table: 1). Hypericin fraction demonstrated high capacity for scavenging the free radicals by reducing the stable 1, 1-diphenyl-2- picrylhydrazyl (DPPH) radical to 1, 1-diphenyl-2-picrylhydrazine (yellow colour) and the reducing capacity increased with increasing concentration of the extract. The IC_{50} was found to be 79.05 µg/mL concentration and was compared with standard Ascorbic acid ($C_6H_8O_6$) with IC_{50} 11.98 µg/mL concentration.

Table 1: DPPH˙ radical scavenging activity of hypericin fraction

S. No.	Concentration (µg/mL)	% of inhibition
		DPPH radical
1	20	12.42 ± 0.38
2	40	32.81 ± 0.19
3	60	47.95 ± 0.35
4	80	50.62 ± 0.38
5	100	58.53 ± 0.65
6	120	59.36 ± 0.49

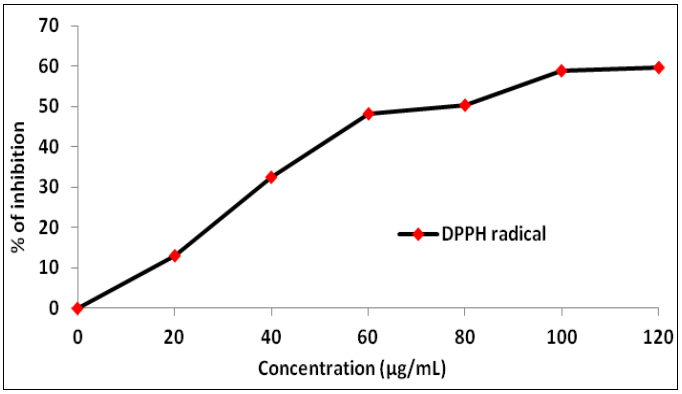


Fig 1: Graph representing DPPH radical scavenging activity of hypericin fraction

3.1.2 Superoxide ($O_2^{\cdot -}$) radical scavenging activity

In superoxide radical scavenging activity, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan, which is measured at 590 nm in UV-Vis spectrophotometer (Systronics, Spectrophotometer 104). Antioxidants have the capacity to inhibit the blue NBT formation and the decrease of absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The maximum superoxide radical scavenging activity of hypericin fraction was 36.05 ± 0.09% at 120 µg/mL concentration (Fig. 2), (Table: 2) and the IC_{50} was found to be 166.41 µg/mL concentration. It was compared with the standard of ascorbic acid ($C_6H_8O_6$) of IC_{50} = 9.65 µg/mL concentration. Later effects of $O_2^{\cdot -}$anion is very harmful to cellular components. The production of other kinds of free radicals and oxidizing agents can be magnified. NBT system will reduce the superoxide anions which are derived from dissolved oxygen by the riboflavin-light-NBT system.

Table 2: Superoxide ($O_2^{\cdot -}$) radical scavenging assay of hypericin fraction

S. No	Concentration (µg/mL)	% of inhibition
		Superoxide radical
1	20	5.68 ± 0.18
2	40	12.58 ± 0.18
3	60	21.28 ± 0.24
4	80	26.23 ± 0.33
5	100	28.50 ± 0.38
6	120	36.05 ± 0.09

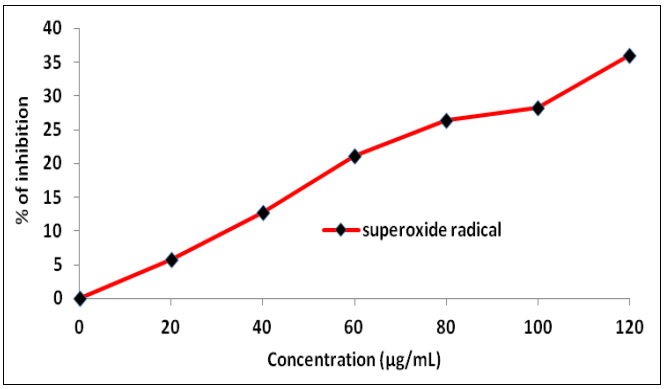


Fig 2: Graph representing superoxide radical scavenging activity of hypericin fraction

3.1.3 Phosphomolybdenum reduction activity

The total antioxidant activity of hypericin fraction was carried out by the phosphomolybdenum reduction method. At acidic pH, Mo (VI) was reduced to Mo(V) by the formation of green phosphate/Mo (V) complex and the maximum absorption of 695 nm. The maximum phosphomolybdenum reduction was measured as 99.71 ± 0.34% at 120 µg/mL concentration (Fig.3),(Table: 3) and the RC_{50} was 10.14 µg/mL concentration. It was compared with the standard ascorbic acid RC_{50} = 6.34 µg/mL concentration. This reduction method helps in investigating the rate of reduction reaction among antioxidant, oxidant and molybdenum ligand. In this quantitative assay, auto-oxidation is generated thermally, when prolonged incubation period at higher temperature is given.

Table 3: Phosphomolybdenum reduction power activity of hypericin fraction

S. No	Concentration (µg/mL)	% of reduction
		Phosphomolybdenum reduction
1	20	53.27 ± 0.99
2	40	79.04 ± 0.27
3	60	88.14 ± 0.38
4	80	92.72 ± 0.41
5	100	97.21 ± 1.05
6	120	98.75 ± 0.35

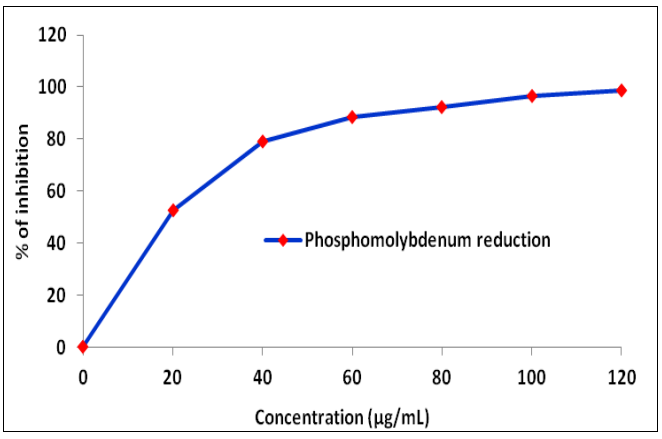


Fig 3: Graph representing phosphomolybdenum reduction activity of hypericin fraction

3.1.4 Ferric (Fe^{3+}) reducing assay

The ferric (Fe^{3+}) reducing power assay is carried out by the reduction of Fe^{3+} to Fe^{2+} by hypericin fraction and thus resulting in the formation of ferro-ferric complex. Increase in concentration of the hypericin fraction, increases the rate of reduction of ferric ions. The maximum Fe^{3+} reduction was found to be 84.81 ± 0.12% at 120 µg/mL concentration (Fig.4), (Table: 4) and the RC_{50} was 20.05 µg/mL concentration. Ascorbic acid was used as the standard with RC_{50} of 7.72 µg/mL concentration. This assay helps in relating absorbance with reduction potential. Higher the absorbance of the reaction mixture, higher is the reduction potential. Depending on the concentration of antioxidants, the colour changes from yellow to green or blue colour represent the reducing capacity of the hypericin fraction.

Table 4: Fe^{3+}reducing power activity of hypericin fraction

S. No	Concentration (µg/mL)	% of Reduction
		Fe^{3+} Reducing Power
1	20	49.86±0.19
2	40	74.73±0.11
3	60	81.47±0.22
4	80	83.09±0.10
5	100	84.61±0.08
6	120	84.81±0.12

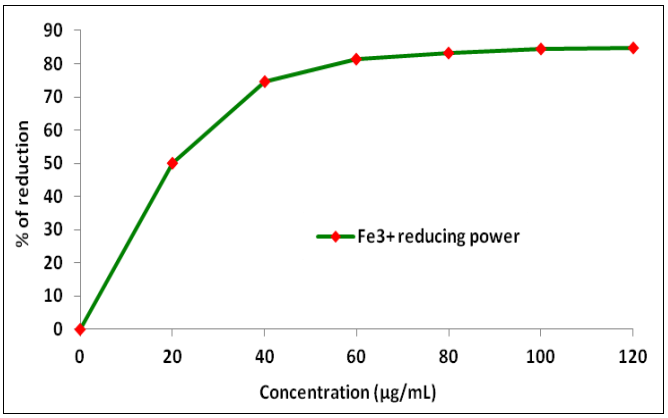


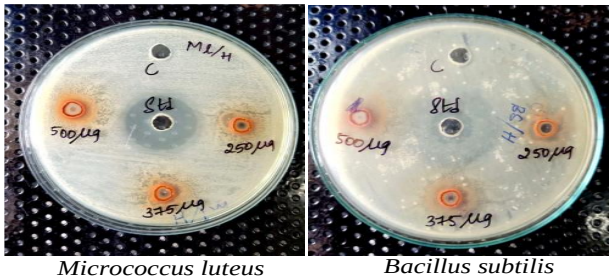
Fig 4: Graph representing Fe^{3+}reduction activity of hypericin fraction

3.2 Antibacterial Activity

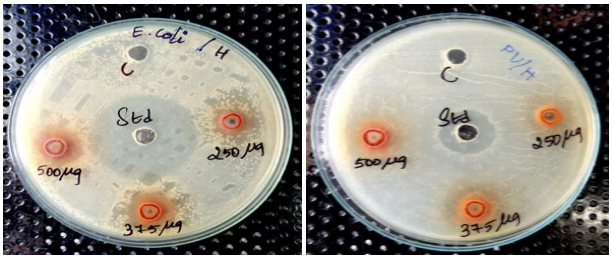
The *in vitro* antibacterial activity of hypericin fraction was performed against both Gram-positive and Gram-negative bacteria. *Micrococcus luteus* and *Bacillus subtilis* as well as *Escherichia coli* and *Proteus vulgaris* were used to carry out the antibacterial assay. The reactivity of hypericin fraction was assessed quantitatively by measuring the diameter of clear zone in cultures in petriplates. The maximum zone of inhibition for effective antibacterial activity against *Bacillus subtilis* was 20 mm and for *Escherichia coli* was 21 mm at 500 µg/mL concentration.

Table 5: Zone of inhibition of varying concentrations of hypericin fraction

Bacterial Organism	Zone of inhibition (mm)			
	250 µg/mL	375 µg/mL	500 µg/mL	Standard Tetracycline (30 µg/mL)
Micrococcus luteus	10	12	13	18
Bacillus subtilis	10	11	12	20
Escherichia coli	12	13	14	21
Proteus vulgaris	12	14	15	14



Micrococcus luteus *Bacillus subtilis*



Escherichia coli *Proteus vulgaris*

Fig 5: Zone inhibition for varying concentrations of hypericin fraction

3.3 Antihaemolytic assay

The erythrocytes of human body have been widely exploited in drug transport. They are considered as the prime targets for reactive oxygen species that attacks, owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA). PUFA and haemoglobin molecules are potent promoters of activated oxygen species mainly target the erythrocytes. Antihaemolytic activity depends majorly, on the concentration of the leaf extract. Moreover, the destruction of RBC during the blood stage of infection accumulates high levels of toxic free heme in circulation that, in turn, has the ability to induce oxidative stress. The hypericin fraction can act as a strong antihaemolytic agent which is due to the presence of some polyphenolic components. The IC_{50} value was found to be 142.38 µg/mL (Fig. 6), (Table: 6) and the maximum antihaemolytic activity of hypericin fraction is 42.14 ± 0.24 µg/mL concentration.

Table 6: Antihaemolytic activity of hypericin fraction

S.NO	Concentration (µg/mL)	Antihaemolytic activity
1	20	0.83 ± 0.094
2	40	2.50 ± 0.24
3	60	9.61 ± 0.39
4	80	12.47 ± 0.53
5	100	42.1 ± 0.46
6	120	42.14 ± 0.26

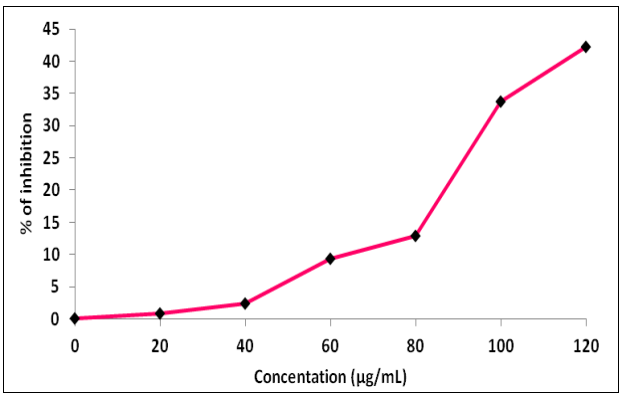


Fig 6: Graph representing Antihaemolytic activity of hypericin fraction

3.4 Thin Layer Chromatography

Thin layer Chromatography was executed in the solvent system of acetone: chloroform: methanol with the ratio of 0.1: 0.15: 0.75. Thin Layer chromatography is one of the most versatile and flexible techniques used for the separation of organic components. The compounds that get separated from the hypericin fraction of leaves extract of *Hypericum hookerianum* and the R_f values were 0.75, 0.79 and 0.86 is clearly represented in the Figure 7.

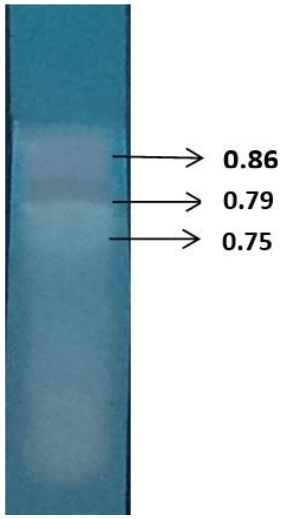


Fig 7: Compounds separated by Thin Layer Chromatography

3.5 Angiogenic activity

Development of blood vessels from in situ differentiating endothelial cells (EC) is called vasculogenesis, whereas sprouting of new blood vessels from the pre-existing ones is termed angiogenesis or neovascularisation. All blood vessels are lined with endothelial cells that must proliferate to form new vessels, migrate to reach remote targets, and survive to limit attrition and senescence. On the other hand the host microenvironment must convey signals for cells to multiply and avoid apoptosis. Angiogenesis is even more critical in cardiovascular disease. Various angiogenic agents are in clinical trials for treating ischaemic heart disease. The formation of new vessels by tissue engineering holds promises to regenerate vessels for cardiac collateralisation and in vascular healing. The *in vivo* angiogenic activity of hypericin fraction was examined in seven day old eggs showed the formation of new blood vessels provides a route for oxygen and nutrient delivery, as well as a conduit for components of the inflammatory response during the healing of wounds (Fig. 8).

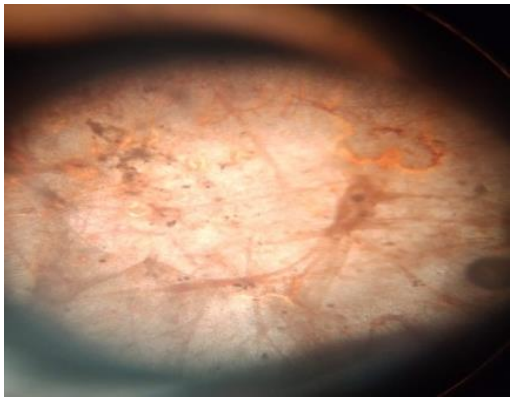
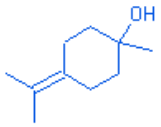
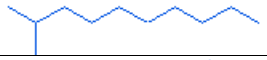
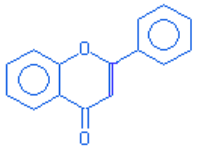
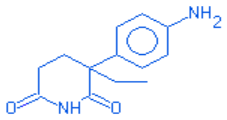
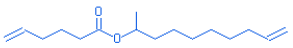
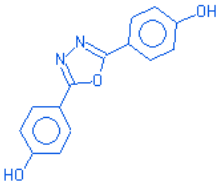
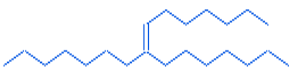
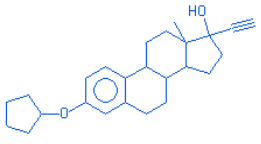


Fig 8: Microscopic view representing the angiogenic property of hypericin fraction

3.6 GC MS analysis

The results obtained from Gas Chromatography- Mass Spectroscopy analysis were represented below (Table: 7). Peaks obtained in GC-MS analysis was represented in the graph (Fig. 9)

Table 7: Components obtained from GC-MS analysis of leaves extract of *Hypericum hookerianum*

S. No	Compound Name	Molecular Formula	RT (Retention Time)	Molecular Weight	Compound Structure
1.	Cyclohexanol, 1 –methyl-4-(1-methylethylidene)-	$C_{10}H_{18}O$	11.45	154.249	
2.	Decane, 2-methyl-	$C_{11}H_{24}$	13.03	156.308	
3.	Flavone	$C_{15}H_{10}O_2$	13.77	222.238	
4.	(+-)-3-(p-Aminophenyl)-3-ethyl-2,6-piperidinedione	$C_{13}H_{16}N_2O_2$	14.57	232.28	
5.	5-Hexenoic acid, (9-decen-2-yl) ester	$C_{16}H_{28}O_2$	15.27	252.007	
6.	2,5–Bis(4- hydroxyphenyl)-1,3,4-oxadiazole	$C_{14}H_{10}N_2O_3$	15.42	254.24	
7.	1 –Eicosene	$C_{20}H_{40}$	15.87	280.531	
8.	11 –Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$	16.95	296.487	
9.	Heptadecanoic acid, 15- methyl-, methyl ester	$C_{19}H_{38}O_2$	17.17	298.503	
10.	Cyclotetracosane	$C_{24}H_{48}$	19.58	336.637	
11.	Pentadecane, 8-heptylidene-	$C_{22}H_{44}$	17.8	308.584	
12.	Quinestrol	$C_{25}H_{32}O_2$	21.87	364.5	

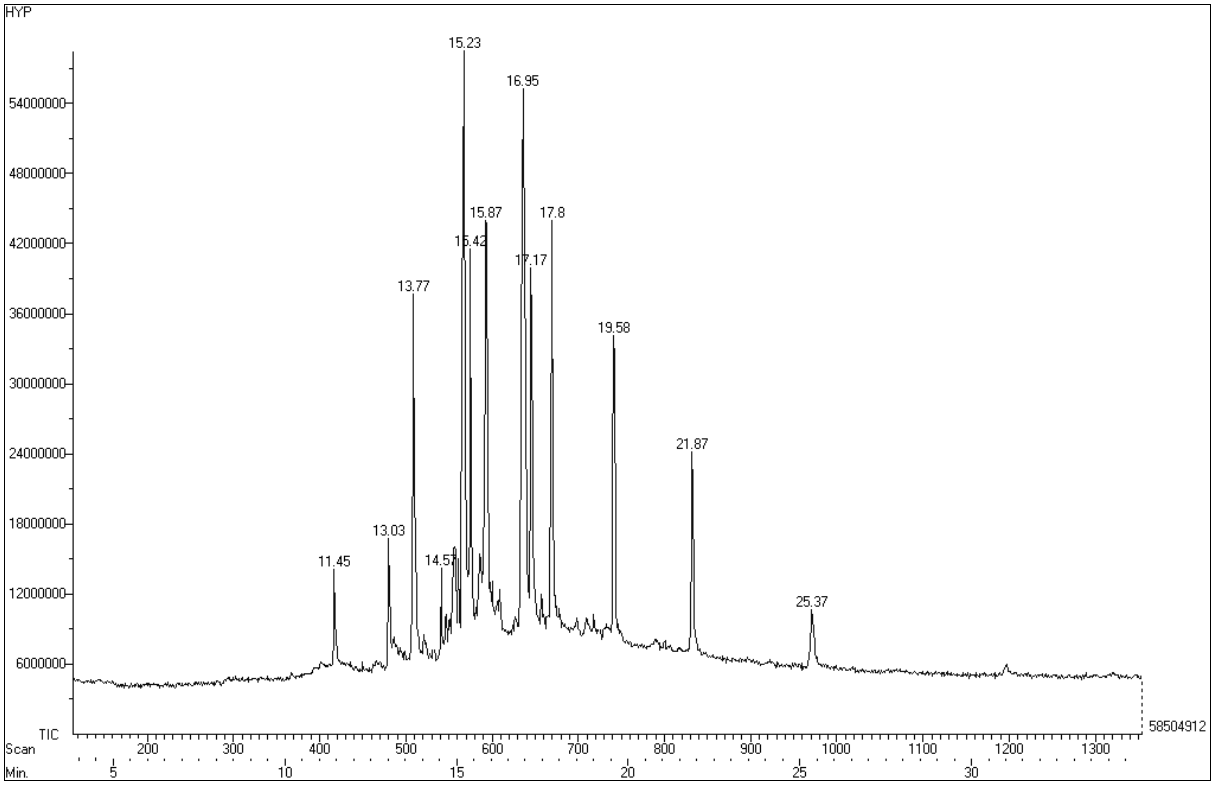


Fig 9: Graph representing the peaks obtained from GC-MS analysis of hypericin fraction

4. Conclusion

Results of the present study shows that hypericin fraction has substances that significantly prevent or slow down the oxidation of an oxidisable substrate particularly in low concentrations and are known to be antioxidants. As we know, plants are potential sources of vital antioxidants, the leaf extract of *Hypericum hookerianum* showed significant antioxidant activities to reduce harmful effect of radicals. The leaf extractof *Hypericum hookerianum* showed effective antibacterial activity against *Micrococcus luteus* and *Proteus vulgaris*. The results of the present study assurethat hypericinfractions can be used as an antioxidant, antibacterial and antihaemolytic agent. The result also proves that the leaf extract of *Hypericum hookerianum* has angiogenic property.

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