Results and Discussion

4.0 RESULTS AND DISCUSSION

Medicinal plants are used to maintain and promote healthy life, prevent disease and cure ailments. It has been estimated that even today, 80% of the world population rely on herbal traditional medicine for their primary health care. Traditional medicinal plants are often cheaper, locally available and easily consumable, raw or as simple medicinal preparations. Nowadays, traditional medicinal practices form an integral part of complementary or alternative medicine (Badugu *et al.*, 2012). Herbal medicines are assumed to be of great importance in the primary healthcare of individuals and communities in many developing countries. For thousands of years, these natural plant products have been utilized for human healthcare in the form of drugs, antioxidants, flavours, fragrances, dyes, insecticides and pheromones. However, during the last century the use of synthetic drugs led to a decline in the use of plant-derived compounds, so that the synthetic drugs would perhaps completely replace the use of traditional plant-derived medicines (Yusuf *et al.*, 2013).

Medicinal plants are abundantly available throughout the world. Medicinal plants are now more focused than ever because they have the capability of producing many benefits to society indeed to mankind, especially in the line of medicine (Shah *et al.*, 2011). In the present study, two different varieties of *Piper betle L.* namely Karpoori and Vellaikodi were subjected to *in vitro* and *in silico* analysis to evaluate the antioxidant and thrombolytic potential of the selected plant. The findings are discussed under the following headings:

4.1. PHYTOCHEMICAL ANALYSIS

4.1.1. QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

The aqueous and ethanolic extract of *Piper betle L.* was prepared and analyzed for the presence or absence of bioactive components in the plant samples. The results are interpreted in Table I.

TABLE I

Phytochemical analysis of *Piper betle L.*

S.No	Phytochemicals	Karpoori		Vellaikodi	
		Aqueous	Ethanol	Aqueous	Ethanol
1	Carbohydrates	+	+	+	+
2	Amino acids and proteins	+	+	+	+
3	Phenols	+	+	+	+
4	Sterol and steroids	-	+	-	+
5	Saponins	+	+	+	+
6	Glycosides	+	+	+	+
7	Alkaloids	+	-	+	-
8	Flavonoids	+	+	+	+
9	Tannins	+	+	+	+
10	Volatile oil	+	+	+	+

'+'→Presence '-'→Absence

The above table indicates that the phytoconstituents such as carbohydrates, amino acids, proteins, phenols, saponin, glycosides, flavonoids, tannins, alkaloids and volatile oil were present and steroids were absent in the aqueous extracts of both variety. In the ethanolic extracts of the plant samples all the above phytochemicals were present except alkaloids. The presence of phytochemicals in the *Piper betle L.* confirms their potential as medicinal plant and also indicates that the two different extracts namely aqueous and ethanol vary in their phytoconstituents.

The results are in agreement with Maobe *et al.*, 2013 who reported that the *Urtica dioica* and *Toddalia asiatica* leaf extracts have saponins, tannins, steroids, terpenoids and flavonoids while alkaloids, cardiac glycoside and anthraquinones were absent. Gard *et al.*, 2013 have revealed the presence of carbohydrate, amino

acids, alkaloids, flavonoid, phenolic compounds and terpenoids in the leaf extract of *Ziziphus oenoplia.*

Kavit *et al.*, 2013 have indicated the presence of medicinally active constituents like tannins, alkaloids, terpenoids, steroids, saponins and the absence of flavonoids, phlobatannins and glycosides in the leaves of *Phyllanthus fraternus.*

4.1.2. QUANTITATIVE ANALYSIS OF PHYTOCONSTITUENTS OF *Piper betle L.*

The quantitative estimation of phytoconstituents was carried out in both Karpoori and Vellaikodi varieties of *Piper betle L.* The results are indicated in Table II and Figure II.

TABLE II

Phytoconstituents of *Piper betle L.*

S.No	Phytoconstituents (mg/g)	Karpoori	Vellaikodi
1.	Flavonoids	48.94 ± 0.63	72.19 ± 1.24
2.	Tannins	59.59 ± 9.59	78.77± 4.79
3.	Protein	64.21 ± 12.51	91.75 ± 6.25

Values are mean ± SD of triplicates

Flavonoids and terpenoids mainly present as colouring pigments in plants also function as potent antioxidants at various levels. Some reports showed that flavonoids could protect membrane lipids from oxidation (Hossain *et al.*, 2006). Tannins are one of the major phytoconstituents found in many higher plants. Tannins are phenolic compounds present in various parts of the plants including the leaves, roots and fruits. (Bennick *et al.*, 2002).

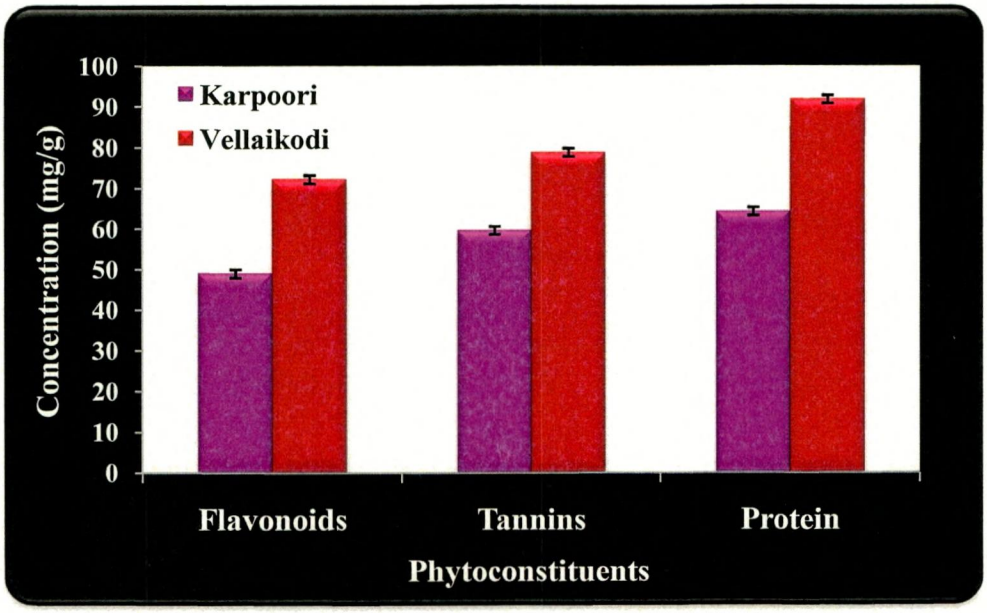
From the table, it is evident that the flavonoid content in both varieties of *Piper betle L.* was found to be 48.94± 0.63 mg/g and 72.19 ± 1.24 mg/g respectively. The tannin content of the plant extracts was found to be 59.59 ± 9.59 mg/g and 78.77 ± 4.79 mg/g. Khatiwora *et al.*, 2010 reported that the

Ipomoea carnea leaf extract contains 84 mg/g of flavonoid content. Tamilselvi *et al.*, 2012 have shown that *Indigofera aspalathoides* methanolic leaf extract contains 34.59±1.788 mg/g of tannin content.

The concentration of Protein in both the extracts was found to be 64.21 ± 12.51 mg/g and 91.75 ± 6.25 mg/g respectively. Khandelwal *et al.*, 2011 suggested that the total levels of protein were found to be 87.8 ± 0.79 mg/g and 54.2 ± 0.77 mg/g in the leaves of *Hamelia patens* and *Petrea volubilis.* Proteins are the molecular instruments through which genetic information is expressed. Hydrolyzing proteins and estimating the amino acids alone will give the exact quantification (Pushpa *et al.*, 2011).

FIGURE II

Phytoconstituents of *Piper betle L.*



4.2. DETERMINATION OF ENZYMIC AND NON-ENZYMIC ANTIOXIDANTS OF *Piper betle L.*

The antioxidant potential of *Piper betle L.* was determined in terms of enzymic and non-enzymic antioxidants.

4.2.1. Enzymic antioxidants

Table III and Figure III indicate the activity of catalase, peroxidase, superoxide dismutase, polyphenol oxidase, glutathione s- transferase and glutathione peroxidase in the aqueous extracts of *Piper betle L.*

TABLE III

The level of enzymic antioxidants of *Piper betle L.*

Samples	Catalase (U*/g)	Peroxidase (U*/g)	Superoxide dismutase (U*/g)	Polyphenol oxidase (U*/g)	Glutathione s-transferase (U*/g)	Glutathione peroxidase (U*/g)
Karpoori	0.67 ± 0.002	0.69 ± 0.006	0.08 ± 0.004	0.03 ± 0.003	0.29 ± 0.003	0.018 ± 0.003
Vellaikodi	0.68 ±0.002	0.73 ± 0.004	0.14 ± 0.003	0.07 ± 0.005	0.35 ± 0.002	0.042 ± 0.007
CD ($p<0.05$)	0.0034	0.0073	0.0080	0.0073	0.0077	0.0086

Values are mean± SD of triplicates

* - Significant at 5% level

U* - Amount of enzyme required to decrease the absorbance by 0.05 units at 240nm.

U* - Unit change in absorbance/ min/g of sample.

U* - Amount of enzyme required to inhibit 50% of NBT reduction.

U* - Amount of enzyme that transforms 1 µmole of dihydrophenol to 1 mole of quinones/min.

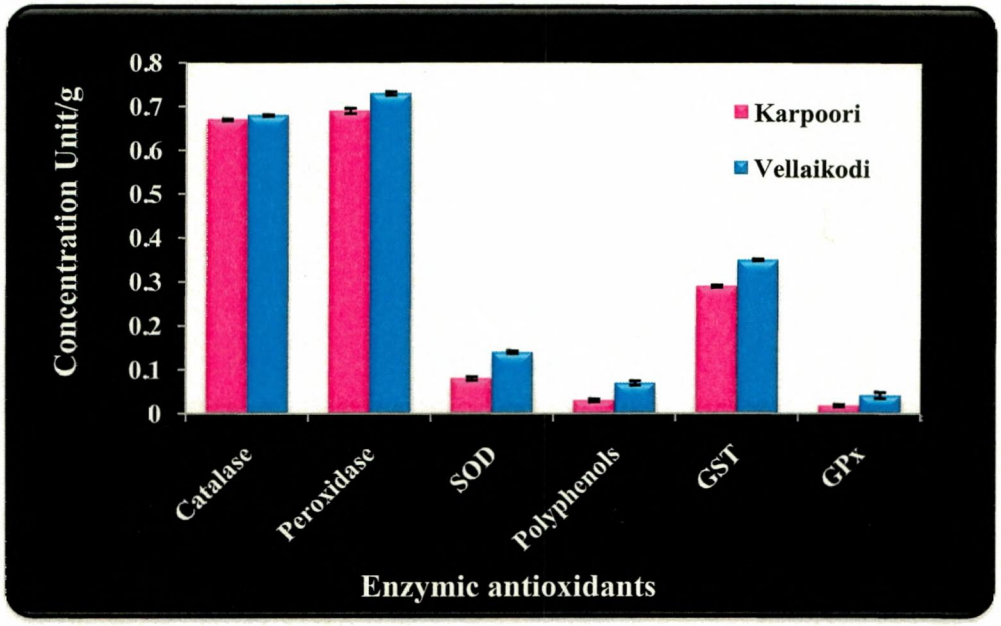
U* - µmoles of CDNB-GSH conjugate per minute per gram sample.

U* - n moles of GSH utilized/min/g protein.

From the above table it is evident that both varieties of Karpoori and Vellaikodi exhibited maximum activity of peroxidase and minimum activity of glutathione peroxidase. The activity of all the enzymic antioxidants was found to be greater in Vellaikodi variety than Karpoori suggesting Vellaikodi as the rich source of enzymic antioxidants.

FIGURE III

The level of enzymic antioxidants of *Piper betle L.*



Plants have developed an array of defense strategies (antioxidant system) to cope up with oxidative stress. The function of this antioxidant system is to scavenge the toxic radicals produced during oxidative stress and thus help the plants to survive through such conditions (Mandal *et al.*, 2009).

Plants have evolved various enzymatic and non enzymatic defense mechanisms. Catalase is one of several antioxidant defense enzymes (SOD, Peroxidase, Glutathione peroxidase) that catalyses dismutation of Hydrogen peroxide to water and oxygen (Beulah *et al.*, 2013). ROS detoxification agents in cells include antioxidative enzymes such as ascorbate oxidase, peroxidase, catalase and ascorbate peroxidase (Gomathi *et al.*, 2012). Battu and Kumar (2012) have shown that the leaves of *Aerva lanata* have antioxidant properties which provide a basis for the traditional use of the plant.

4.2.2. Non-enzymic antioxidants

Table IV and Figure IV represent the level of vitamin C, vitamin E, polyphenols and reduced glutathione in the aqueous extracts of *Piper betle L.*

TABLE IV

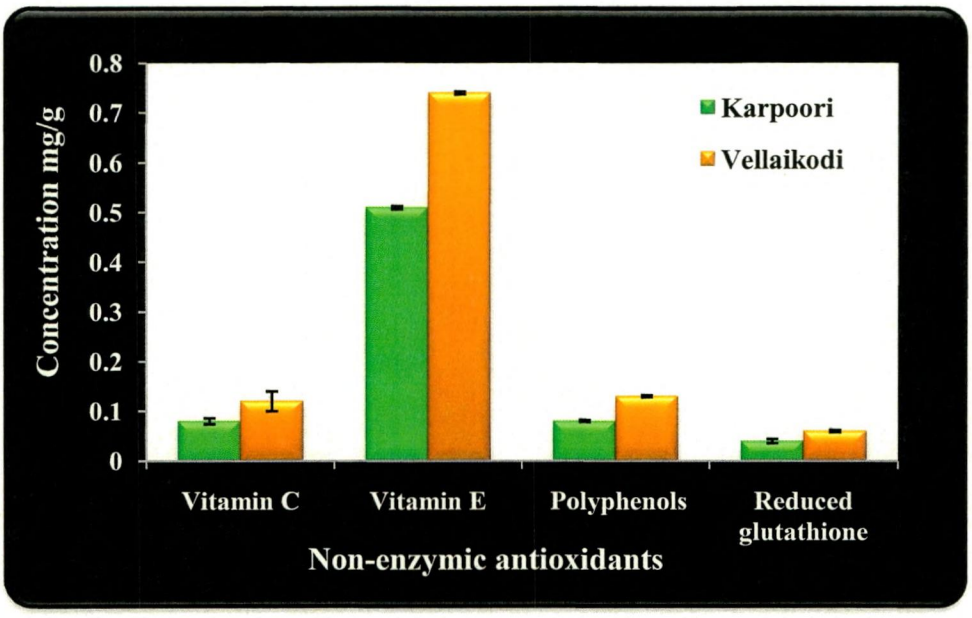
The level of non- enzymic antioxidants of *Piper betle L.*

Samples	Vitamin C (mg/g)	Vitamin E (µg/g)	Polyphenols (mg/g)	Reduced glutathione (n moles/g)
Karpoori	0.08 ± 0.006	0.51 ± 0.003	0.08 ± 0.002	0.04 ± 0.004
Vellaikodi	0.12 ± 0.020	0.74 ± 0.003	0.13 ± 0.002	0.06 ± 0.002
CD ($p<0.05$)	0.0249	0.0464	0.0030	0.0052

Values are mean± SD of triplicates

FIGURE IV

The level of non-enzymic antioxidants of *Piper betle L.*



The results revealed that Vitamin E was significantly higher than other antioxidants in both Karpoori and Vellaikodi varieties of *Piper betle L.* and the value was 0.51 ± 0.003µg/g and 0.74 ± 0.003 µg/g respectively. Vellaikodi variety recorded higher values for all the non-enzymic antioxidants than Karpoori variety.

Veeru *et al.*, 2009 screened crude extracts of *Desmodium gangeticum, Eclipta alba, Ocimum sanctum, Piper longum, Solanum nigrum* and *Amaranthus*

caudatus for antioxidant activity on the basis of their scavenging effect and reported that the overall antioxidant activity of *D. gangeticum* was found to be the strongest, followed in descending order by *A. caudatus, S. nigrum, P. longum, E. alba* and *O. sanctum*.

Also, *D. gangeticum* recorded high quantity of ascorbic acid and *A. caudatus* registered the least value. In the present study, Vellaikodi variety recorded higher values for both enzymic and non-enzymic antioxidants. Thus the study reveals that both varieties of *Piper betle L.* exhibited good antioxidant activity and the results suggest that the selected plants can be used as a source of antioxidants for pharmacological preparations.

4.3. TOTAL ANTIOXIDANT CAPACITY OF *Piper betle L.*

The total antioxidant activity was calculated in terms of Gallic acid (mg/g) and the results are given in Table V and Figure V.

TABLE V

Total antioxidant capacity of *Piper betle L.*

Concentration of the extract (mg/ml)	Total antioxidant capacity (mg/g)	
	Karpoori	Vellaikodi
5	154.8 ± 5.14	423.2 ± 2.59
10	229.4 ± 4.5	602.1 ± 2.59
15	269.6 ± 4.45	937.6 ± 6.82

Values are mean ± SD of triplicates

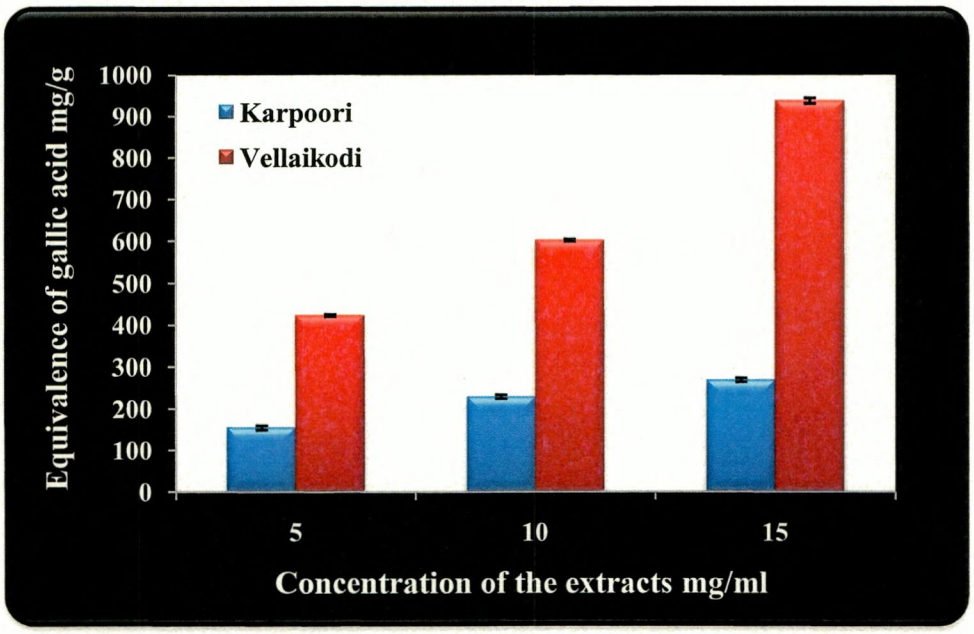
The above table indicates that both the Karpoori and Vellaikodi varieties of *Piper betle L.* exhibited an increasing trend in the total antioxidant activity with increase in concentration. The aqueous extract of Vellaikodi variety exhibited a greater antioxidant activity than Karpoori variety.

Abbasi *et al.*, 2013 found that, the aqueous soluble fraction had total antioxidant activity of 0.152±0.041 mg/g than other soluble fractions of *Euphorbia heterophylla* Linn. plant extract.

Thambiraj *et al.*, 2012 showed that, the total antioxidant activity was determined to be 42.18 ± 0.67 mg/ g for the methanolic leaf extracts of *Acacia caesia* which is comparable to that of the standard, ascorbic acid (30.12 mg/g).

FIGURE V

Total antioxidant capacity of *Piper betle L.*



4.4. THROMBOLYTIC ACTIVITY OF AQUEOUS EXTRACTS OF *Piper betle L.*

Thrombolytic activity of aqueous extracts of Karpoori and Vellaikodi variety was determined using human blood. A series of concentration 250, 500 and 1000µg/ml were used and the percent clot lysis was determined. The values are tabulated as in Table VI and are represented as in Figure VI.

TABLE VI

Thrombolytic activity of aqueous extracts of *Piper betle L.*

Samples	Concentration (µg/ml)	% Clot lysis		
		Control (Normal persons) (n=10)	Diabetic patients (n=10)	Hypertensive patients (n=10)
Karpoori	250	17.02 ± 5.56	20.40 ± 5.87	29.84 ± 9.64
	500	19.63 ± 5.93	27.01 ± 6.36	36.51 ± 7.00
	1000	22.79 ± 5.44	33.07 ± 4.96	40.00 ± 8.05
Vellaikodi	250	18.99 ± 3.94	21.21 ± 4.36	32.37 ± 7.05
	500	22.53 ± 4.41	25.49 ± 3.56	34.77 ±6.16
	1000	25.39 ± 4.66	31.43 ± 3.96	39.00 ± 6.65
Positive control (Clavix)	1000	29.76 ± 8.36	35.11 ± 8.60	47.67 ± 8.96
Negative control (Water)	1000	15.76 ± 6.48	16.43 ± 5.45	23.67 ± 8.22
SEd CD (p<0.05)		2.74209 5.44770	2.66148 5.28754	3.56154 7.07568

Values are mean ± SD of three triplicates

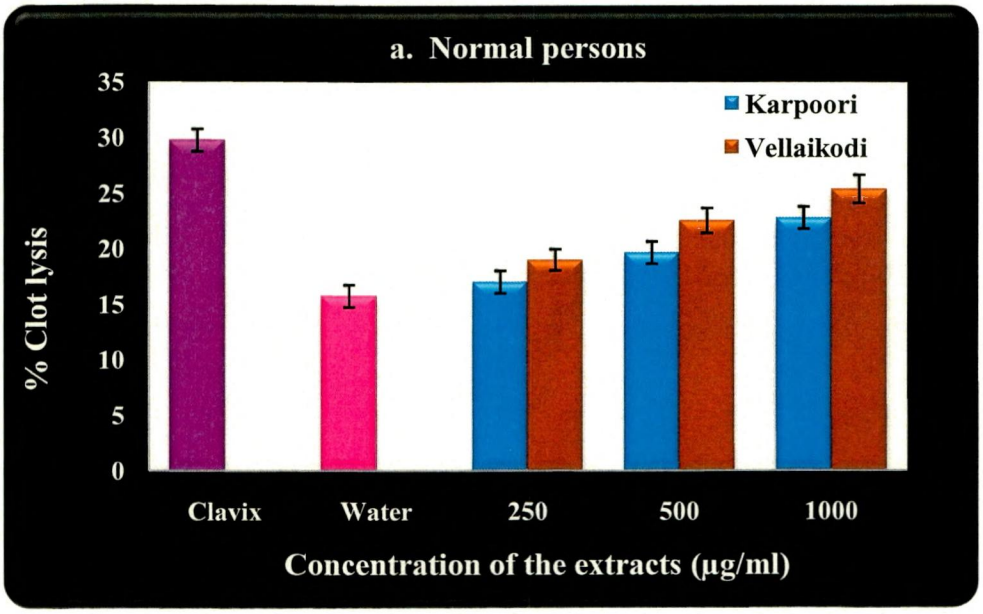
The table represents that both the varieties of *Piper betle L.* exhibited a proportional increase in clot lysis with increase in concentration. The maximal clot lysis was observed at a concentration of 1000µg/ml in both the varieties and it was comparable to that of the positive control. The negative control (water) exhibited a minimum % clot lysis in both the varieties of the plant samples. Comparison of % clot lysis among the three different groups revealed that the percentage clot lysis was found to be increased in diabetic and hypertensive patients but the hypertensive patients exerted a maximal value. Of the two varieties of betle leaves Vellaikodi recorded greater % of clot lysis.

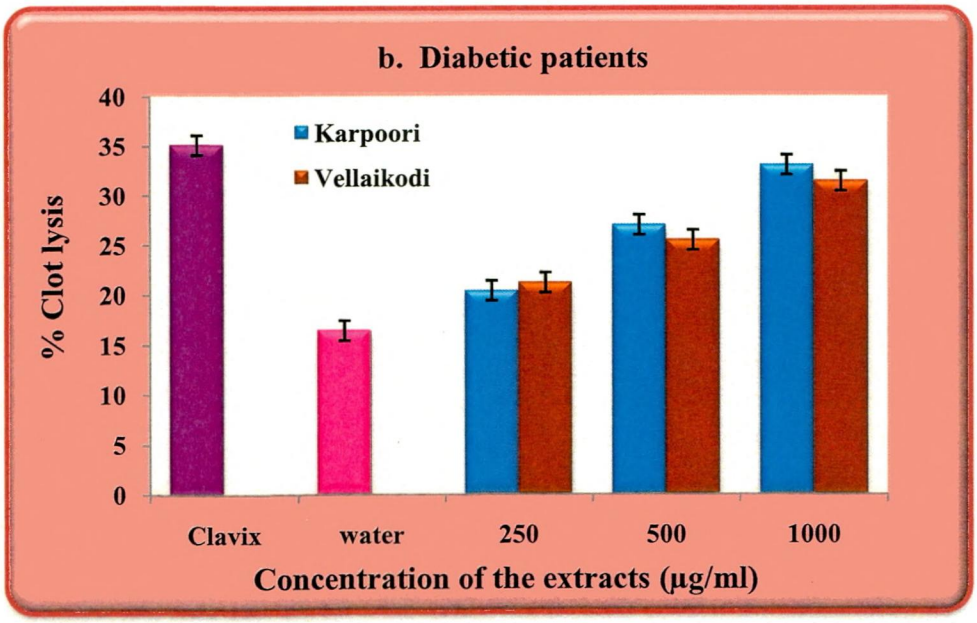
The investigation of thrombolytic activity of *Bougainvillea spectabilis* leaf extract by Sherwani *et al.*, 2013 showed maximum 84. 24% clot lysis at 800 µg/ml concentration in 72 hrs of incubation and clearly indicated that the concentrations of leaf extract enhanced the percentage of clot lysis. Sarker *et al.*, 2012 have

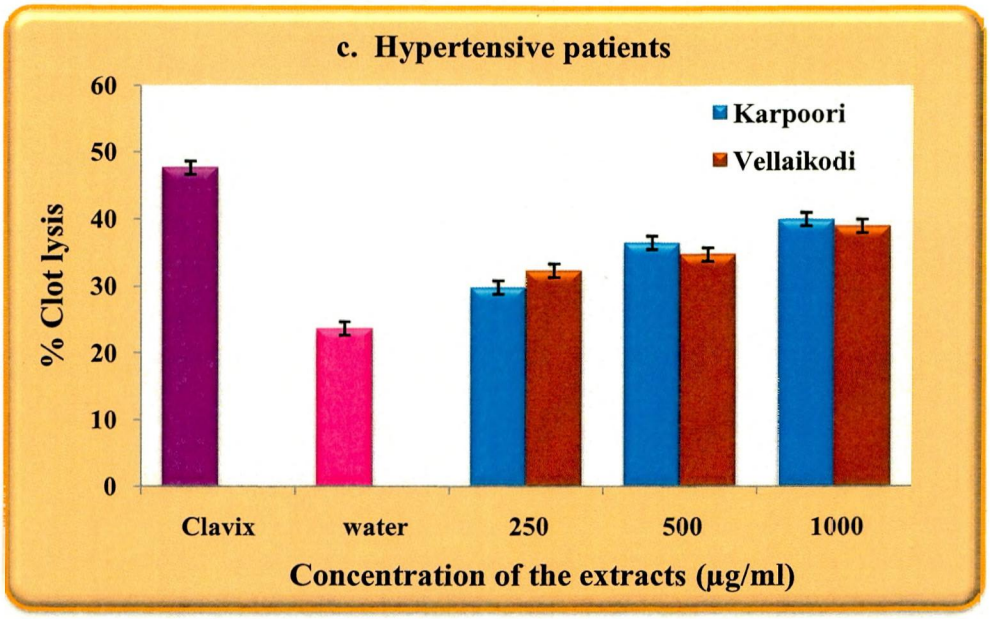
indicated that, in the clot lysis study, the aqueous soluble fractions of *Allamanda cathartica* leaf showed 30.73±0.884 % clot lysis activity, the chloroform soluble fractions showed 34.51 ± 0.669% clot lysis and the hexane soluble fractions showed 32.179 ± 0.581 % clot lysis.

FIGURE VI

Thrombolytic activity of aqueous extracts of *Piper betle L.*







Clavix - (+) control

Water - (-) control

In the present study, the two varieties of betle leaves at a concentration of 1000 µg/ml recorded significant increase in clot lysis when compared to negative control (water). This is in agreement with Hossain *et al.*, 2013 who have shown that there is a significant difference between the clot lysis of aqueous extract of *Camellia sinensis* leaves and the vehicle control.

4.5. COMPARISON OF SERUM CHOLESTEROL LEVEL AND THROMBOLYTIC ACTIVITY OF *Piper betle L.*

Total cholesterol was estimated in serum obtained from blood samples used for clot lysis. A comparison was made between the serum cholesterol level and percent clot lysis in all the three groups such as normal persons, diabetic and hypertensive patients. The values are indicated in Table VII, VIII & IX and Figure VII, VIII & IX.

TABLE VII

Comparison of serum cholesterol level and thrombolytic activity of Normal persons

S.NO	Level of serum cholesterol(mg/dl)	Clot lysis (%)	
		Karpoori	Vellaikodi
1.	103.5	28.99	26.98
2.	123.6	11.45	14.49
3.	130.0	26.86	25.64
4.	158.4	13.78	23.33
5.	167.3	16.65	19.55
6.	185.5	17.88	21.29
7.	200.0	20.43	24.84
8.	228.8	19.61	19.37
9.	256.4	18.64	27.05
10.	307.7	23.75	20.35

TABLE VIII

Comparison of serum cholesterol level and thrombolytic activity of Diabetic patients

S.No	Level of serum cholesterol(mg/dl)	Clot lysis (%)	
		Karpoori	Vellaikodi
1.	123.7	17.05	25.54
2.	142.9	24.79	27.09
3.	153.0	21.60	28.69
4.	174.4	26.73	30.14
5.	194.0	24.98	25.69
6.	205.0	24.63	25.11
7.	220.6	32.98	35.8
8.	284.4	30.30	28.65
9.	300.0	34.58	29.57
10.	408.0	30.52	30.68

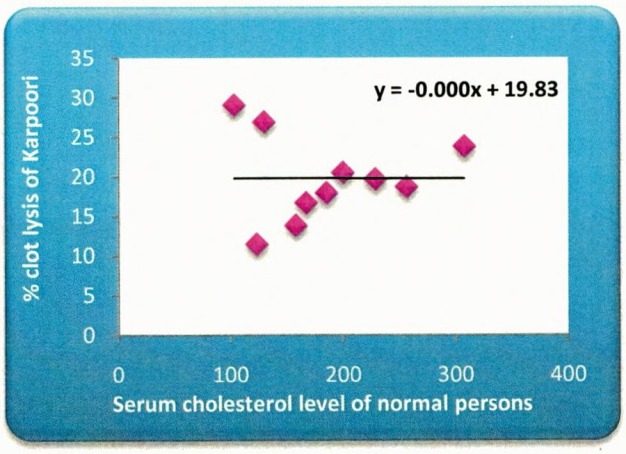
TABLE IX

Comparison of serum cholesterol level and thrombolytic activity of Hypertensive patients

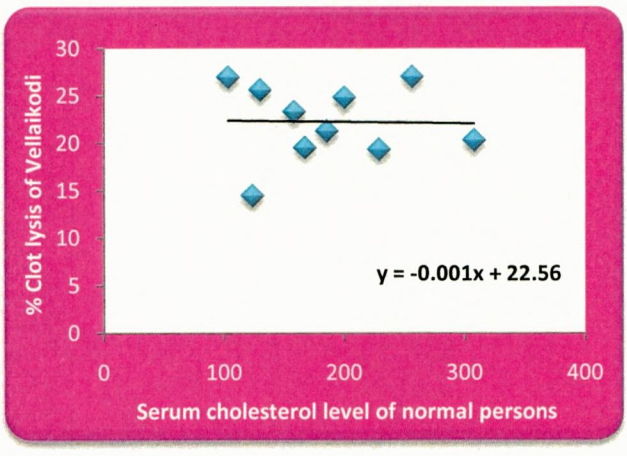
S.No	Level of serum cholesterol(mg/dl)	Clot lysis (%)	
		Karpoori	Vellaikodi
1.	108.5	24.81	32.82
2.	124.0	35.12	22.43
3.	130.6	33.92	32.80
4.	152.9	44.02	47.99
5.	168.9	39.75	40.72
6.	175.4	29.02	32.66
7.	219.0	39.13	36.27
8.	320.5	31.85	47.13
9.	348.7	27.27	43.52
10.	400.0	30.26	36.59

FIGURE VII

Comparison of serum cholesterol level and thrombolytic activity of Normal persons



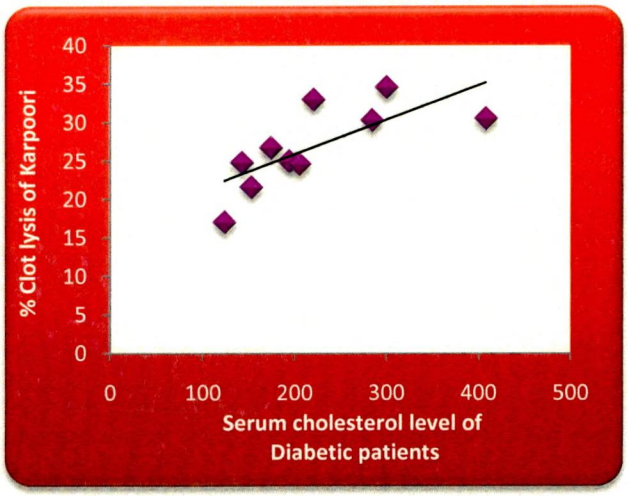
R=5E-06** – Significant at 5%

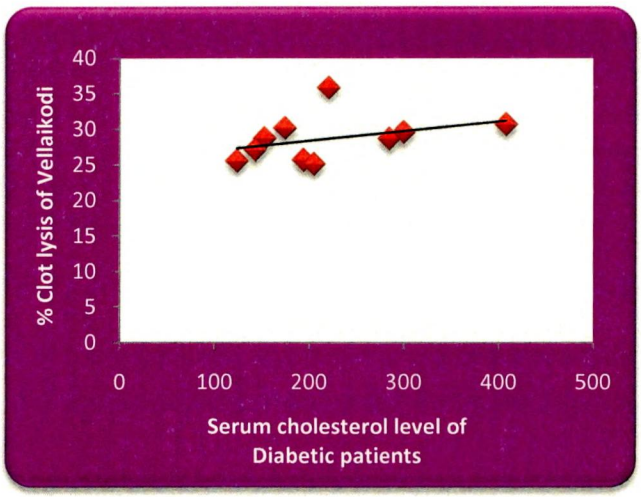


R= 0.001^{ns} ns – Not significant at 5%

FIGURE VIII

Comparison of serum cholesterol level and thrombolytic activity of Diabetic patients



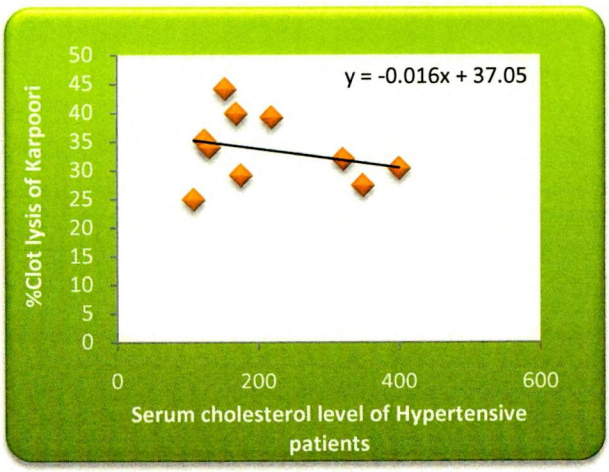


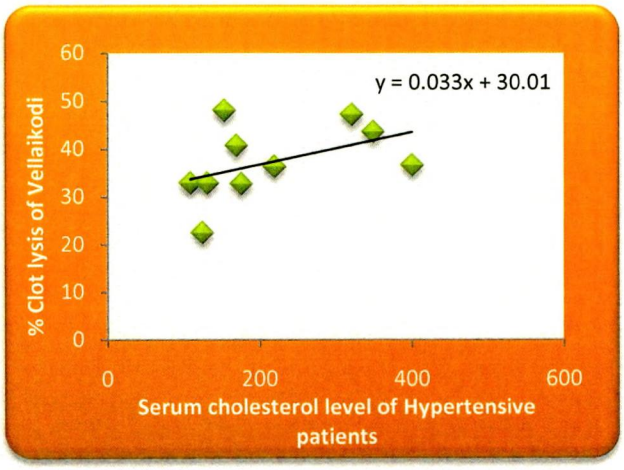
R=0.532** – Significant at 5%

R=0.140** – Significant at 5%

FIGURE IX

Comparison of serum cholesterol level and thrombolytic activity of Hypertensive patients





R= 0.079ns ns – Not significant at 5%

R= 0.205* * – Significant at 5%

Correlation analysis was carried out by comparing the serum cholesterol level and percentage clot lysis by the plant extracts. The results revealed that in case of diabetic patients, there is positive correlation between the level of serum cholesterol and thrombolytic activity of both the varieties of betle leaves and the values are significant at 5% level. In the case of normal and hypertensive persons comparison of serum cholesterol level and % clot lysis indicated that Karpoori exhibited positive correlation in normal persons and Vellaikodi showed a positive correlation in hypertensive persons.

4.6. CYTOTOXICITY SCREENING OF AQUEOUS EXTRACTS OF *Piper betle L.*

Brine shrimp lethality assay was carried out to determine the cytotoxic effect of plant extracts. Percentage lethality of *Artemia salina* was determined after 24 hours of exposure to the plant extracts and the findings are represented in Table X & XI and Figure X & XI.

TABLE X

Cytotoxicity screening of aqueous extracts of *Piper betle L.*

S.No	Concentration (µg/ml)	Percentage Lethality	
		Karpoori	Vellaikodi
1.	0	0	0
2.	200	10	5
3.	400	25	15
4.	600	35	30
5.	800	50	45
6.	1000	85	70
LC_{50} (µg/ml)		800	888.90

Values are mean of triplicates

TABLE XI

Cytotoxicity screening of Potassium Dichromate (positive control)

S.No	Concentration of potassium dichromate (µg/ml)	Percentage Lethality
1.	0	0
2.	5	15
3.	10	20
4.	15	30
5.	20	43
6.	25	62
7.	30	75
8.	35	86
9.	40	95
LC_{50} (µg/ml)		23

Values are mean of triplicates

The results of cytotoxicity screening are expressed in percentage lethality. The effect of varying concentrations on the mortality of brine shrimps revealed that % mortality increased with increase in concentration suggesting a direct proportional relationship between the concentration of the extracts and the degree of lethality.

Aqueous extracts of Karpoori and Vellaikodi varieties exhibited LC_{50} value of 800µg/ml and 888.90µg/ml respectively. Potassium dichromate was used as a positive control which showed LC_{50} value of 23µg/ml. Vellaikodi showed higher Cytotoxic effect when compared to Karpoori variety. Comparison of % lethality of the plant extracts and the positive control showed that the betle leaves are less toxic. Jethinlalkhosh *et al.*, 2013 found that the active fraction of both aqueous and methanolic extracts of *Terminalia arjuna* showed prominent results in brine shrimp cytotoxic assay in a dose-dependent manner.

FIGURE X

Cytotoxicity screening of aqueous extracts of *Piper betle L.*

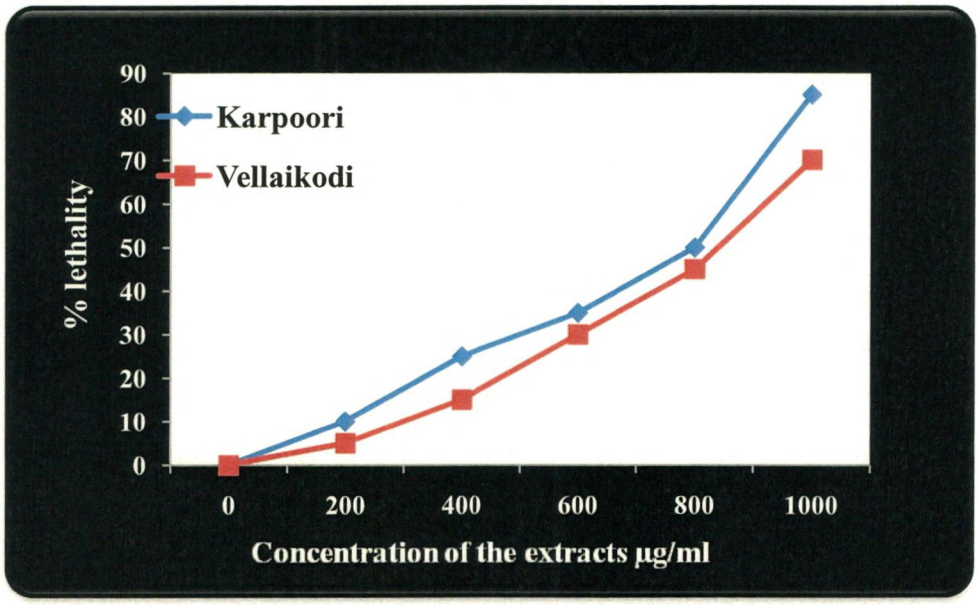
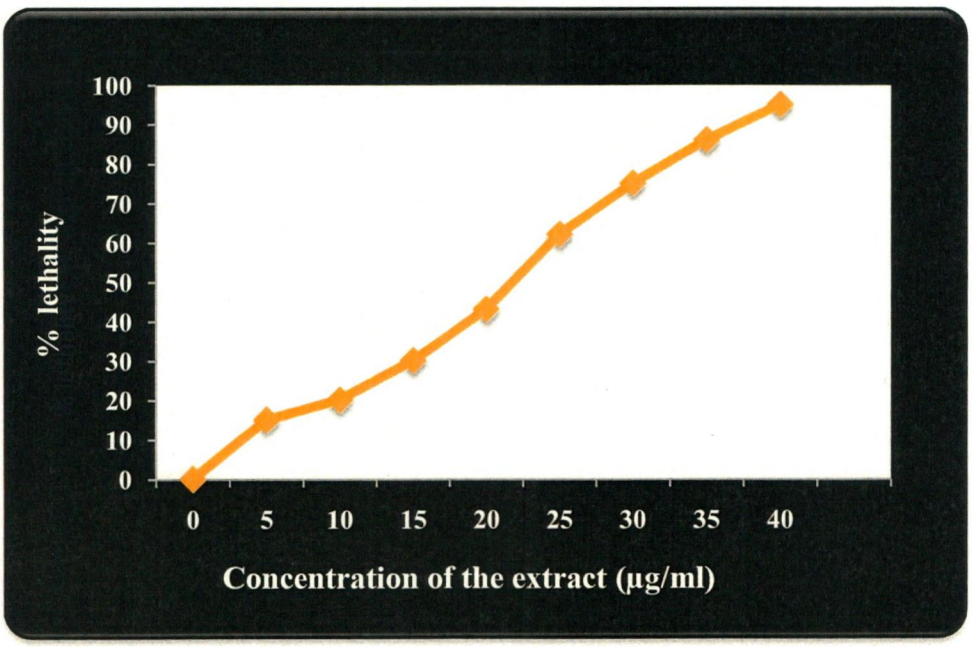


FIGURE XI

Cytotoxicity screening of Potassium Dichromate (positive control)



The brine shrimp lethality bioassay is used as a screening tool for the determination of bioactivity of different extracts, fractions and pure compounds. This test is an indication of Cytotoxicity, anticancer, antiviral, pesticidal, antimicrobial and other different pharmacological activities. The brine shrimp lethality bioassay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and antitumor properties (Sharma *et al.*, 2013).

4.7. HPTLC PROFILING OF *Piper betle L.*

High Performance Thin Layer Chromatography (HPTLC) was performed to quantify the secondary metabolites of phytocomponents present in the plant extracts. The screening of flavonoids and tannins by HPTLC profiling was done using methanolic extracts of both the varieties of *Piper betle L.* The results are interpreted in Table XII & XIII, Plate II & III and Figure XII and XIII.

TABLE XII

Peak table for Flavonoid profile of methanolic extracts of *Piper betle L.*

Track	Peak	Rf	Height	Area	Assigned substance
Sample 1	1	0.07	33.6	539.8	Unknown
Sample 1	2	0.13	13.8	168.6	Unknown
Sample 1	3	0.44	16.3	262.7	Unknown
Sample 1	4	0.71	52.6	2556.0	Unknown
Sample 1	5	0.79	37.7	1001.0	Unknown
Sample 2	1	0.07	249.8	4919.0	Unknown
Sample 2	2	0.14	58.6	1865.4	Flavonoid 1
Sample 2	3	0.19	32.5	1191.7	Unknown
Sample 2	4	0.52	22.9	361.2	Unknown
Sample 2	5	0.64	124.0	5344	Flavonoid 1
Sample 2	6	0.67	161.6	4219.2	Unknown
Sample 2	7	0.71	49.3	2996.1	Unknown
STD	1	0.66	215.0	6051.6	Flavonoid standard

Sample 1 – Karpoori **Sample 2** – Vellaikodi

PLATE II

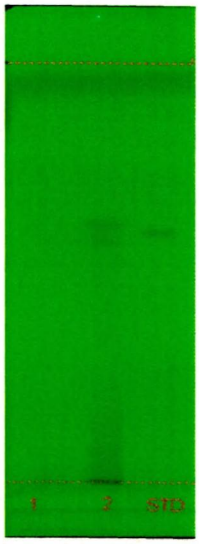
Chromatogram of methanolic extracts of *Piper betle L.* (Flavonoids)

Before derivatization

Visible light

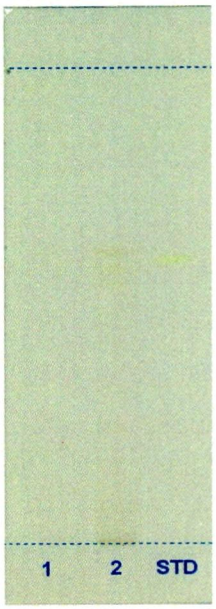
UV 366nm

UV 254nm



After derivatization

Visible light



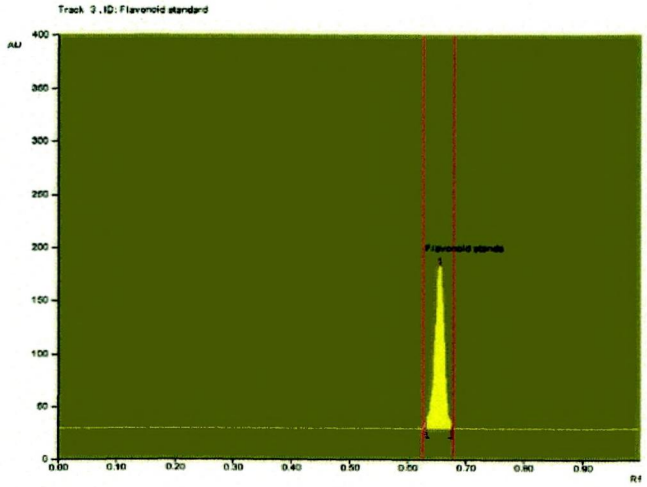
UV 366nm

From Plate II, it is indicated that the Yellow, Yellowish blue coloured fluorescent zone at UV 366nm mode were present in the tracks. Chromatogram after derivatization confirmed the Presence of Flavonoids.

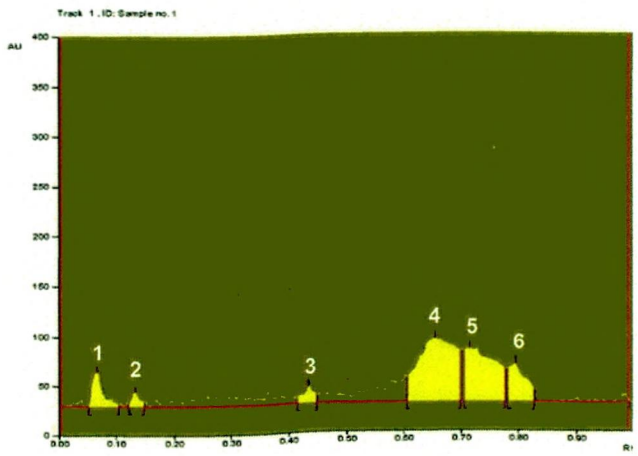
FIGURE XII

Peak densitogram of Flavonoids

Flavonoid standard



Methanolic extract of Karpoori



Methanolic extract of Vellaikodi

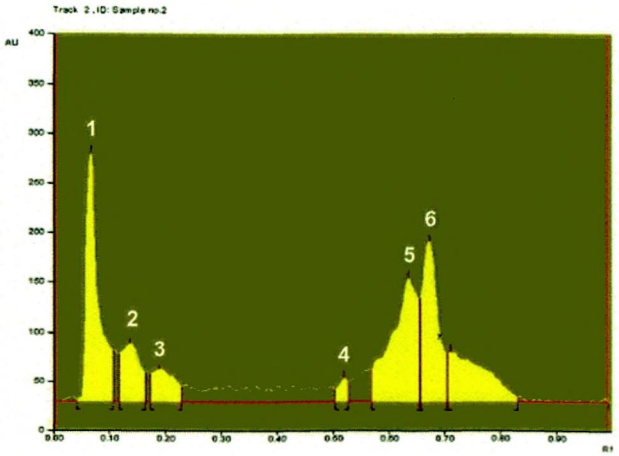


Table XII and Figure XII showed the flavonoid profile of the methanolic extracts of *Piper betle L.* The flavonoid standard exhibited Rf value of 0.66. In this profile,flavonoid compound was present in the methanolic extracts of *Piper betle L.* with Rf value of 0.14 and 0.64for Vellaikodi variety. Peak 2 and 5in the methanolic extract of Vellaikodi variety chromatogram confirmed the presence of flavonoid which matches with the peak present in the chromatogram of the Flavonoid standard.

TABLE XIII

Peak table for Tannin profile of methanolic extracts of *Piper betle L.*

Track	Peak	Rf	Height	Area	Assigned substance
Sample 1	1	0.03	12.0	146.9	Unknown
Sample 1	2	0.07	74.9	1025.7	Unknown
Sample 1	3	0.61	13.6	263.0	Unknown
Sample 1	4	0.91	87.7	6858.9	Unknown
Sample 2	1	0.03	16.5	264.5	Unknown
Sample 2	2	0.07	263.1	4941.1	Unknown
Sample 2	3	0.15	19.6	424.4	Unknown
Sample 2	4	0.42	28.6	961.5	Unknown
Sample 2	5	0.63	44.9	952.0	Tannin 1
Sample 2	6	0.77	55.3	2264.6	Unknown
STD	1	0.41	116.4	3661.6	Tannin standard

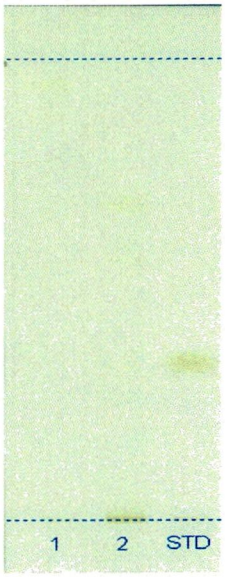
Sample 1 – Karpoori **Sample 2** - Vellaikodi

PLATE III

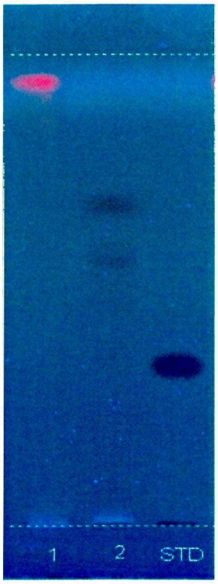
Chromatogram of methanolic extracts of *Piper betle L.* (Tannins)

Before derivatization

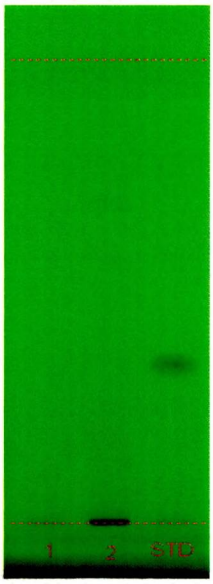
Visible light



UV 366nm

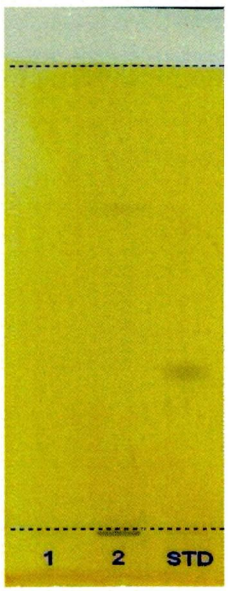


UV 254nm



After Derivatization

Visible light

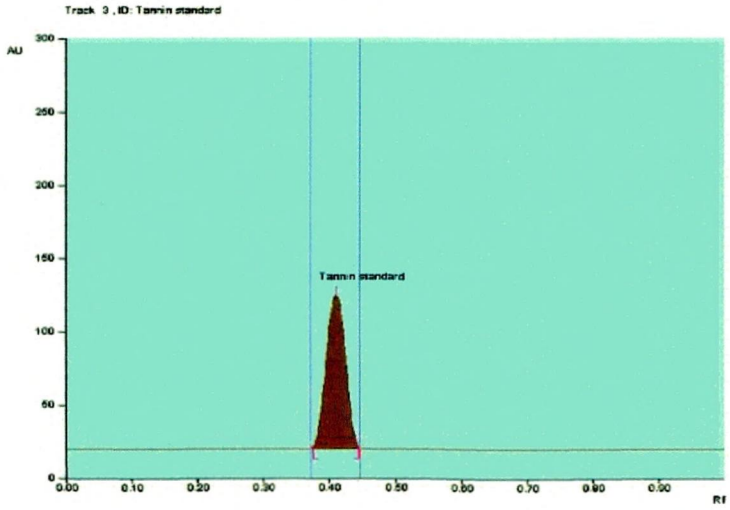


From Plate III, it is evident that the brown, greenish brown coloured zone at Visible light mode were present in the tracks. Chromatogram after derivatization confirmed the Presence of Tannins.

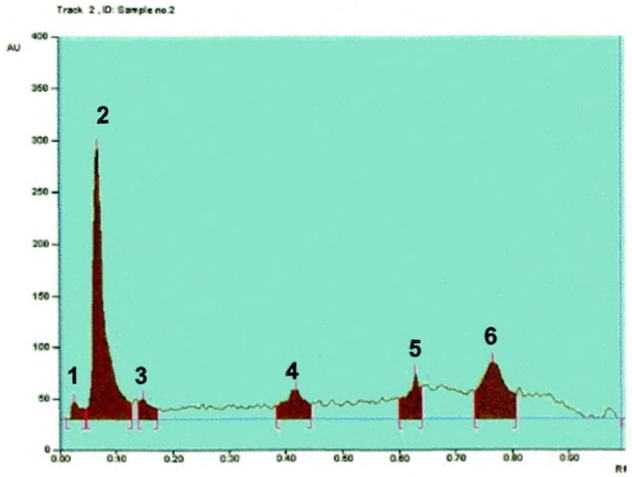
FIGURE XIII

Peak Densitogram of Tannins

Tannin standard



Methanolic extract of Vellaikodi



Methanolic extract of Karpoori

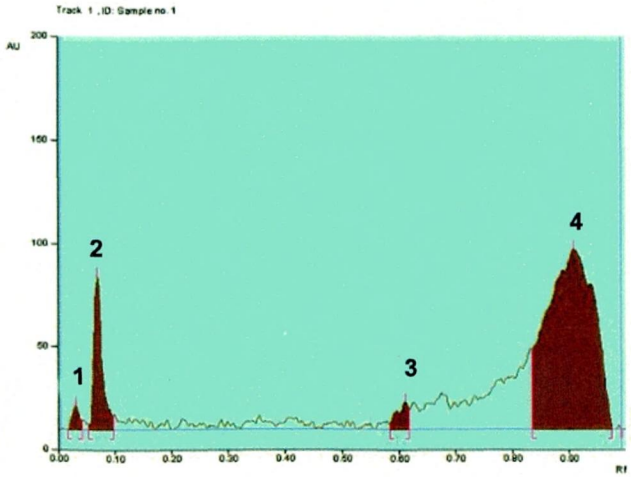


Table XIII and Figure XIII showed the tannin profile of the methanolic extracts of *Piper betle L.* The tannin standard exhibited Rf value of 0.41. In this profile, tannin compound was present in the methanolic extracts of *Piper betle L.* with Rf value of 0.63 for Vellaikodi variety. Peak 5 in the methanolic extract of Vellaikodi variety chromatogram confirmed the presence of tannin which matches with the peak present in the chromatogram of the tannin standard.

Devi *et al.*, 2012 indicated that nine different types of tannins have been observed in the leaves of *Aerva lanata*. Among the nine different tannins of leaves, tannin with Rf values 0.30, 0.63 and 0.72 are unique and they are not present in other aerial parts of the plant.

4.8. DOCKING STUDIES

In silico screening of the ligands and/or of the receptors has become an essential tool to facilitate the drug discovery process but compound collections are needed to carry out such *in silico* experiments. The compound hydroxychavicol was subjected to *in silico* studies using Schrodinger drug design suite version 9.3 for their efficacy against the target proteins present in blood clotting factors. The target proteins such as tissue factor VIIa (4JYU), VIII (1WBG) and X (2Y7X) were selected for the docking studies. The 3D structures of the target proteins were obtained from the Protein Data Bank and the structures were refined using the protein preparation wizard module. The molecular docking and ADME studies were performed to characterize the active components.

4.8.1. ADME studies

ADME (Absorption, Distribution, Metabolism and Excretion) profile of hydroxychavicol was determined by QikProp. QikProp 3.0 module of Schrodinger predicts physically significant descriptors and pharmaceutically relevant properties of organic molecules, either individually or in batches. The QikProp result of the ligand, hydroxychavicol is represented in Table XIV and XV.

TABLE XIV

ADME results of the ligand using QikProp

S.No	Descriptors	Standard Values	Ligand Values
1	Molecular weight (Da)	130.0-725.0	150.177
2	Number of hydrogen bond donors	0.0/6.0	2.000
3	Number of hydrogen bond acceptors	2.0-20.0	1.500
4	QP log P for octanol/ water	-2.0/6.5	0.951
5	Apparent Caco-2 Permeability (nm/sec)	<25 poor, >500 Great	1521
6	Apparent MDCK Permeability (nm/sec)	<25 poor, >500 Great	778
7	Lipinski Rule of 5 Violations	(maximum is 4)	0
8	% Human Oral Absorption in GI (±20%)	(<25% is poor)	89%
9	Qualitative Model for Human Oral Absorption	(>80% is high)	High

The QikProp results for hydroxychavicol showed that the compounds satisfy the Lipinski rule of five, and the percentage of human oral absorption in gastrointestinal tract is 89. The lipophilicity is expressed as the partition coefficient P in octanol/water, which indicates good absorption and distribution. Therefore, the ligand was then subjected to docking using Glide.

4.8.2. Molecular docking using glide

The ligand, hydroxychavicol was docked to the proteins using Glide in standard precision mode and the Glide score was calculated. For the prediction of results, four parameters were considered namely G-score, Glide energy, H-bonds and Good contacts, which indicate the binding affinity of the ligand towards the receptor.

The target proteins in blood clotting factors were tissue factor VIIa, VIII and X. The affinity of hydroxychavicol with the target proteins and top ranked poses generated by Glide SP docking are given in Table XV.

TABLE XV

Glide SP docking of the Hydroxychavicol with the target proteins Tissue factor VIIa, VIII and X

Descriptors	Ligand docked with		
	VIIa	VIII	X
Glide score	-5.561	-5.348	-5.872
Energy (kcal/mol)	-44.2	-36.39	-43.37
Good contacts	175	205	171
Pose number	146	278	16
Number of H- bonds	2	3	1
Conformation number	3	2	3

The above table represents that hydroxychavicol showed a good glide score of -5.561, -5.348 and -5.872with the target proteins TF VIIa, VIII and X respectively. It also showed a stronger interaction with TF-VIII target protein through H-bonds. The docking efficiency and the molecular interactions showing good contacts of hydroxychavicol with the target proteins TF VIIa, VIII and X are represented in Figure XIV, XV, XVI, XVII, XVIII and XIX.

FIGURE XIV

Docking of hydroxychavicol with Tissue factor VIIa

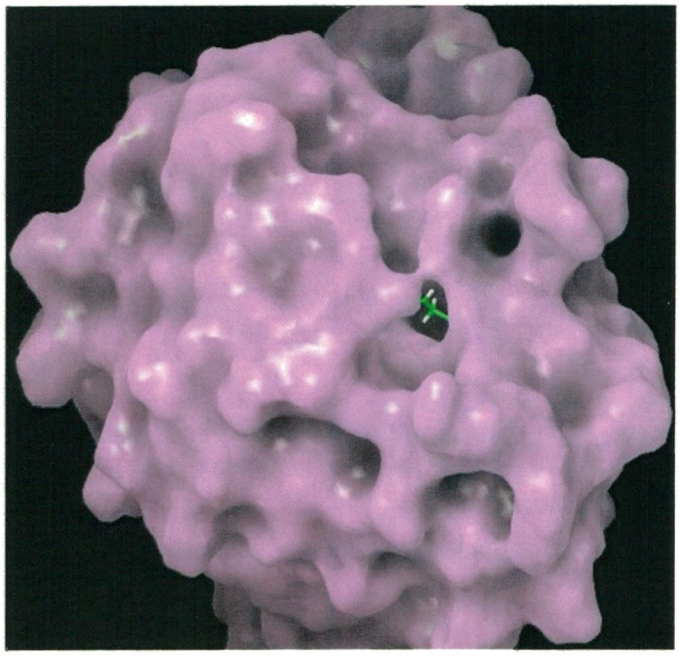


FIGURE XV

Molecular interaction of TF VIIa with hydroxychavicol showing good contacts and H-bonds

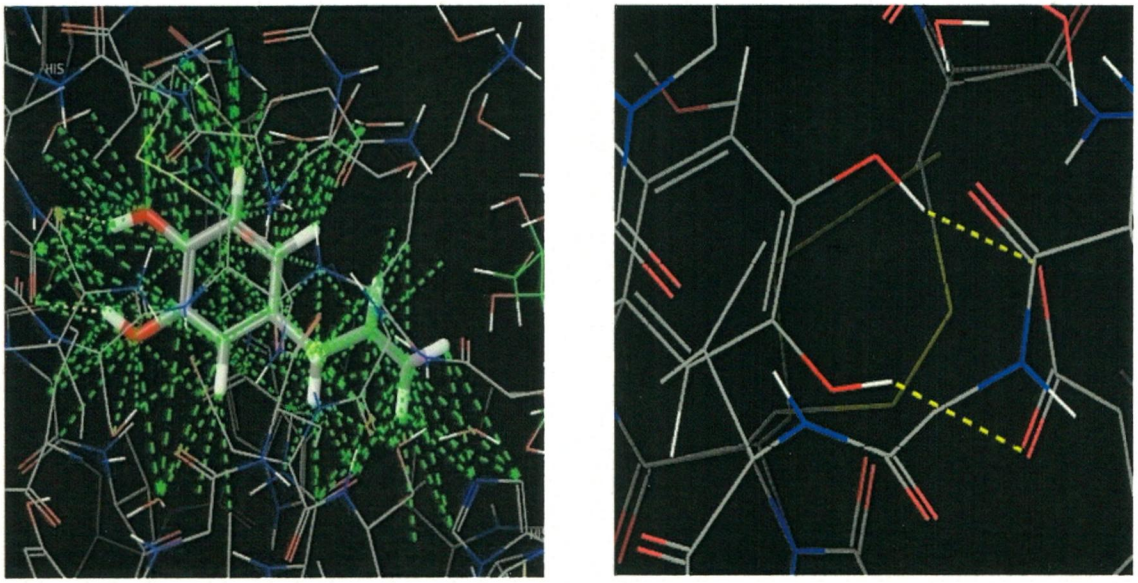


FIGURE XVIII

Docking of hydroxychavicol with Tissue factor X

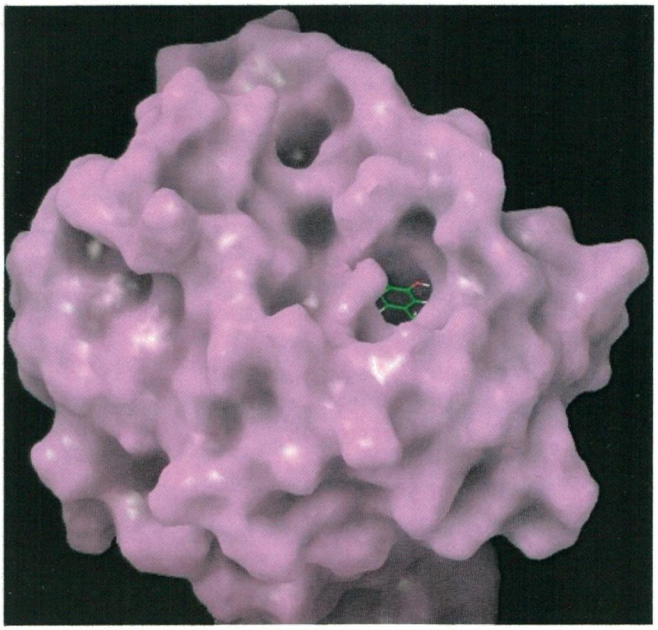
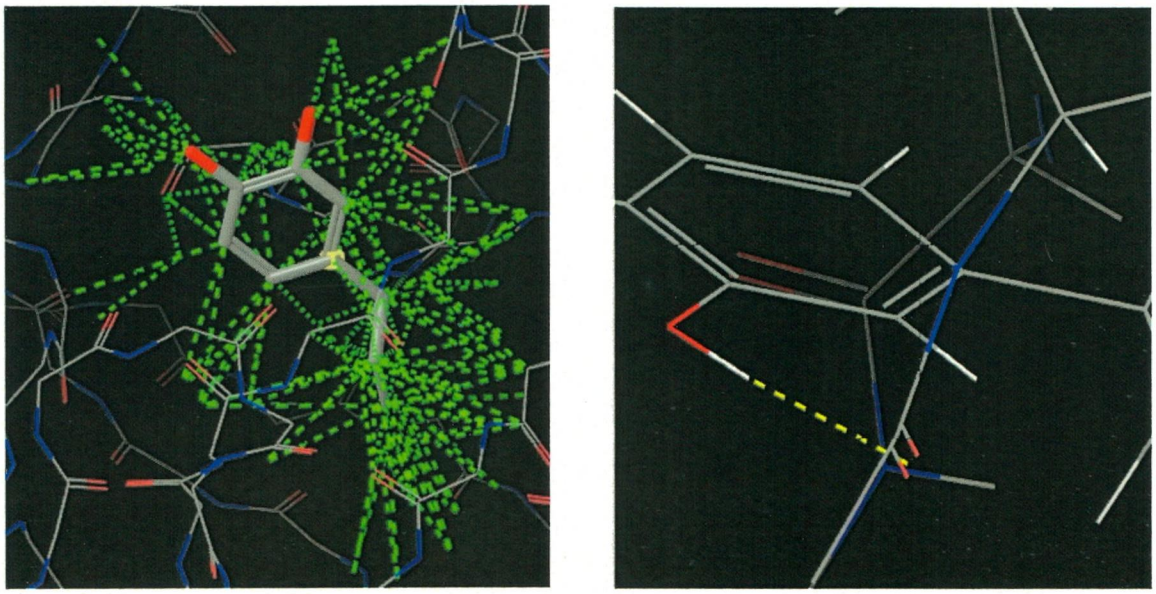


FIGURE XIX

Molecular interaction of TF X with hydroxychavicol showing good contacts and H-bonds



The compound hydroxychavicol was selected to analyse its efficacy against blood clotting factors in humans. It can be concluded that hydroxychavicol possessed good docking scores and high binding affinity to the proteins associated with the clotting process. The bioavailability of the compound was supported by the ADME profile. The *in silico* studies indicated the interaction of hydroxychavicol with the target proteins namely TF VIIa, VIII and X helps in the prevention of bleeding disorders. This interaction plays a vital role in evaluating the thrombolytic effect.

The above results are in agreement with the report of Balavignesh *et al.*, (2013) wherein the analysis of *Acacia concinna* plant exhibited docking score and energy. The ligand Palmitic acid showed the best results than other ligands namely methyl palmitate, linolenic acid, geranyl acetone and methyl linoleate suggesting palmitic acid as the effective inhibitor for the inhibition of hepatitis C virus.

Docking studies represent the binding mode within all of the inhibitors and the target protein and finally brings forth the bioactive conformation of molecules (Gabrielsen *et al.*, 2012). Gunda *et al.*, 2013 carried out the docking study to reveal the information on structural features of Factor- XIII inhibitors and also varied paths to design novel potent analogues with increased activity.