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Evaluation of Antifibrin, Antioxidant and Antimicrobial Activities of Betel Leaves (*Piper betel L.*)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Piper betel L. belongs to the family Piperaceae. It has been an important medicinal agent since ages in various traditional and folk systems of medicine. Leaves obtained from the local market were shade dried and powdered. Different solvents were used based on polarity to extract phytochemicals from this powder using a Soxhlet extractor and separated using rotary vacuum evaporator. Thin layer chromatography was run using different solvent systems in different ratios for identifying essential compounds of *Piper betel* and for standardizing the ratios at which better resolution of compounds taken place. Antimicrobial activities were tested on twelve bacterial and three fungal species. Also, anti fibrin activity was tested on erythrocytes by using the extracts obtained by the plant. The zone of inhibitions formed due to the anti microbial activity were measured and found that mixtures of ethyl acetate and ethanol were effective. The percentage of clot lysis was found to be appreciable for ethyl acetate extract of the Piper leaves.

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1. INTRODUCTION

The use of medicinal plants dates back to many decades as they were found to be promising resources of many antibiotics which have many advantages such as safer and minimum risk of side effects [1]. *Piper betel. L* is one such type which is very helpful in curing many of the diseases caused by bacteria and fungi. This plant is a glabrous climbing vine that belongs to the family piperaceae that is distributed widely in many Asian countries. Many curative properties such as antifertility, antiulcer, antidiabetic, antiplatelet aggregation, antitumor, antimutagenic were identified with this plant [2-10]. Also, the leaf is used to treat many types of diseases such as asthma, leprosy, bronchitis and alcoholism [11]. The plant was used to cure various ailments related to liver fibrosis and carcinoma was also reported to have biological properties such as antioxidation and detoxication [12].

Thrombotic disorders play an important role in increasing the morbidity and mortality rate of human beings. Clot busters from natural sources viz., plant sources would be more effective with minimum side effects, will be more suitable for therapeutic usage. Therefore in the present study, different extracts of *Piper betel* were tested against different types of bacterial and fungal species, as well as for antifibrin properties. Hence, the present work is focused on determining the anti fibrin and anti microbial activities of the betel leaf which can be used potentially to treat various infectious diseases.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Material

Fresh *Piper betel. L* (Piperaceae) leaves were collected from local market, Guntur, Andhra Pradesh. Collected leaves were washed, dried in between filter papers and air dried. The leaves were powdered in a blender and stored at room temperature for further use. The powdered leaves were then subjected to extraction using Ethanol, Ethyl Acetate, Hexane+Petroleum Ether and aqueous in shaking condition. After that the solvents were separated using rotary vacuum evaporator and used to test antimicrobial and anti-fibrin activities.

2.2 Microorganisms and Media

Different microorganisms were used for checking the antimicrobial activities. Test cultures used for the study include bacteria like *Escherichia coli, Bacillus sphaericus, Streptococcus mutans, Lactococus lactis, Proteus mirabilis, Serratia marcescens, Bacillus subtilis, Enterobacter aerogenes, Pseudomonas aeruginosa, Wautersia eutropha, Salmonella typhi, Vibrio cholera,* and fungi like *Aspergillus niger, Saccharomyces cerevisiae, Penicillium chrysogenum* were procured from IMTECH Chandigarh. These bacterial and fungal strains used for study were cultured on respective medium at respective temperatures for 24-48hrs and stored in a refrigerator.

2.3 In vitro Antimicrobial Assay

Antimicrobial activity was checked against the above mentioned bacterial and fungal species. The respective media were prepared, and poured into plates in which freshly prepared bacteria are inoculated and kept for incubation at respective temperatures. After the cultures enter the log phase, wells are made using well punching equipment. Extracts of each 40µl was added into wells and again kept for incubation. After complete growth (24 hours) the plates were checked for Zone of Inhibition and the results were tabulated. The same procedure was followed for fungal cultures using respective media and incubated for 48 hours at their respective temperature all these were performed in triplicates. Results obtained were tabulated.

2.4 Determination of Total Phenolic Content

The phenolic content of the plant extracts was determined by the Folin-Ciocalteu reagent [13]. Each solution of extract (400 µl) was thoroughly mixed with 1ml of FC-reagent. After mixing for 3minutes 1.6 ml of 7.5% (w/v) sodium carbonate was added and allowed to stand for 30minutes in the dark. The absorbance was measured at 765 nm against a blank. The total phenolic content in the different extracts is expressed as Gallic acid

equivalents in milligrams per gram dry weight of the plant. Results obtained were tabulated.

2.5 Determination of Antioxidant Activity

The antioxidant activity of piper betel extracts were determined by three different methods DPPH free radical scavenging activity, reducing activity and hydrogen peroxide scavenging activity. All these assays are carried out in triplicates and average values were considered.

2.5.1 DPPH free radical scavenging activity

The effect of the different extracts of piper betel on DPPH radical (1, 1-diphenil-2-picrydrazyl) was investigated using the method described by limei et al. Equal volumes of diluted extracts were mixed an equal volume of DPPH 6 × 10-3M in absolute ethanol and the absorbance was measured at 517nm against the control [14]. The ability to scavenge DPPH radical was calculated using the formula:

DPPH radical scavenging activity-
$[(Abs_{control} - Abs_{test})/ (Abs_{control})] \times 100$.

2.5.2 Reducing activity

Phosphate buffer of 0.2M was prepared with sodium dihydrogen phosphate and disodium hydrogen phosphate in the ratio 3:2 at pH 6.6 and 1% potassium ferricyanide solution was also prepared. Then buffer and ferricyanide solution were mixed in the ratio 1:1 now 5ml of the mixed solution was added to 2.5 ml of extract of different concentrations (10, 25, 50, and 100) µg/ml. Then the whole mixture was incubated at room temperature for 20 minutes. Again 2.5 ml of 10% trichloroactetic acid was added to stop the reaction. After that the mixture is subjected to centrifugation for 10 minutes at 3000rpm then 2.5ml of supernatant solution was taken and added to 0.5ml of 1% ferric chloride and left for 10 minutes and the absorbance was measured at 230nm against a blank [15].

2.5.3 Hydrogen peroxide scavenging assay

40mM of hydrogen peroxide was prepared in 100ml of phosphate buffer of pH 7.4. Then 0.6 ml of hydrogen peroxide solution was added to 2.5ml of extracts at different extracts at various concentrations (10, 25, 50, and 100) µg/ml and standards were prepared with ascorbic acid at same concentrations and allowed to leave for 10 minutes. After incubation absorbance was measured at 230nm by UV – spectrophotometer [16].

2.6 Determination of Anti Fibrin Activity

1ml of human blood was taken in 6 separate centrifuge tubes, incubated at 25^0c for 20 minutes and centrifuged at 10,000 RPM for 10 min. Supernatant was removed and the weight of clot formed were measured. Different solvent extracts (each 50 µl) were added to four tubes and the 5th tube with distilled water (control) and 6^{th} tube was left undisturbed without any additions. Tubes were kept for incubation at 25^0c for 2 hours. After incubation tubes were again centrifuged and weight of clots was measured. Percentage lysis of fibrin was calculated using the data obtained from the experiments and the results were tabulated. In the same way, anti fibrin activity was also checked at 35°C.

2.7 Separation Method

The obtained mixture was subjected to thin layer chromatography on silica gel plates with hexane and ethyl acetate as solvent mixture in different ratios.

a. Hexane and ethyl acetate in the ratios of 5:5, 6:4, 2:8, 6:3:1(with acetone) respectively for separating the compounds present in the hexane + petroleum ether extract.
b. Hexane and Ethyl acetate in the ratios 5:5, 6:4, 2:8, 6:3:1(with acetone) respectively for separating the components present in the ethyl acetate extract.
c. Hexane and ethyl acetate in the ratios of 5:5, 6:4, 2:8, 6:3:1(with acetone) respectively for separating the components present in the ethanol extract.

The obtained chromatograms were visualized under ultra violet light. Iodine chamber was used to fix the components.

3. RESULTS AND DISCUSSION

3.1 Determination of Anti-Microbial Activity

Natural products are in great demand owing to their invaluable biological properties and bioactive components which have proved to be very useful against large number of diseases. Researchers have extensively studied the

biological properties of *Piper betel. L* and their results showed that this plant is ethno-medically valuable [17,18]. In order to check the antimicrobial activity of extracted plant samples, agar well diffusion method was used. Aqueous, Ethyl Acetate, Hexane and Ethanolic extracts of Piper betel leaves were taken for the present study. Agar well diffusion method used here was performed in accordance to a method reported in similar study carried out by Bari et al. [19].

The aqueous extract of piper betel showed the maximum zone of inhibition (10mm) against *Lactococcus latis* followed by zone of inhibition (09mm) against *Asperigillus niger*. Ethyl Acetate extract of *Piper betel* showed the maximum Zone of Inhibition (18 mm) against *Pseudomonas aeruginosa and Wautersia eutropha,* followed by Zone of Inhibition (17mm) against *Proteus mirabilis*. The Ethanolic extract of *Piper betel* showed the maximum Zone of Inhibition (19mm) against *Escherichia coli,* followed by Zone of Inhibition (16 mm) against *Streptococcus mutans*. The Hexane extract of piper betel showed maximum zone of inhibition (09mm) *Saccharomyces cerevisiae* followed by zone of inhibition (08mm) of *Bacillus sphaericus*. The Ethanol, Hexane, Ethyl Acetate and Aqueous extracts of *Piper betel. L* possessed good antimicrobial properties against several bacteria chosen in the study. The antibacterial activity of leaves of *Piper betel* may be indicative of presence of metabolic toxins or broad spectrum antimicrobial compounds that act against gram positive as well as gram negative bacteria especially in solvents Ethanol, Ethyl Acetate and Methanol (Table 1).

3.2 Determination of Total Phenolic Content and Antioxidant Activities of Extracts

The total phenolic content of the extracts was determined and compared. The solvents used for extraction also affected the concentration of total phenolic content in extracts. Ethyl acetate found to be the good solvent system for the extraction of phenolic compounds and it is found to be 69.12 (Table 2).Hexane showed the best Antioxidant activity for all the assays performed (Tables 3, 4 and 5). All other extracts are also having good antioxidant activities and the activity of the extracts increasing gradually with increase in concentration of the extracts. Hence *Piper betel.* L extracts possessed good antioxidant activities which can be used in treating metabolic disorders in near future.

3.3 Determination of Anti Fibrin Activity

Anti fibrin activity for extracts on bacterial and fungal species was done at the temperatures 25°C and 37°C and the results were compared. Ethyl acetate extract showed the maximum lysis of clot at both temperatures i.e. 21.92 and 21.84. the ethanolic extract showed maximum percentage lysis at 37°C when compared at 25°C. Hexane extract also lysed the colt to some extent of 8.62% at 37^0c and 6.09% at 25^0c

Table 1. Antimicrobial activity of different extracts

Organism	Antibiotic (tetracycline)	Aqueous			Ethyl acetate			Ethanol			Hexane		
		A	B	C	A	B	C	A	B	C	A	B	C
Escherichia coli	11	7	6	6	14	11	13	15	19	17	7	9	6
Bacillus sphaericus	5	6	5	8	12	14	14	8	---	11	8	9	8
Streptococcus mutans	9	4	4	7	13	10	12	12	15	16	8	7	5
lactococus lactis	3	11	9	9	9	13	11	11	8	15	7	7	4
Proteus mirabilis	7	5	5	6	14	17	16	14	14	11	7	5	6
Serratia marcescens	4	3	5	3	4	7	---	11	11	13	9	4	8
Bacillus subtilis	5	9	8	7	10	9	11	14	13	12	0	--	2
Enterobacter aerogenes	7	6	7	4	7	13	11	15	11	15	6	7	4
Saccharomyces cerevisiae	2	8	5	7	10	11	11	9	6	10	9	9	8
Wautersia eutropha	7	2	3	2	14	18	16	6	9	9	8	6	5
Salmonella typhi	7	3	2	2	8	6	8	4	7	4	1	1	---
Vibrio cholera	9	2	4	4	8	11	7	6	6	3	3	1	4
Penicillium chrysogenum	3	4	6	6	13	11	13	11	15	13	0	3	4
Pseudomonas aeruginosa	2	7	6	6	18	16	17	9	12	14	7	5	7
Asperigillus niger	4	9	9	9	---	---	5	--	7	11	3	9	9

results obtained were tabulated and compared (Tables 6 and 7) (Graph 4). The lysis of colt was also checked for aqueous extract and distilled water. No significant percentage lysis was observed. Therefore it is clearly visible that the extracts of Piper betel. L possessed good anti fibrin activity which can be attributed to the fact that this wonder leaf with more research can be used for the treatment of thrombotic disorders in near future.

3.4 Separation Method- Thin layer Chromatography

Thin layer chromatography was performed for better resolution of plant components and were found to be 6:3:1 (with acetone) and 5:5. The compounds of Hexane + petroleum ether and ethyl acetate extracts resolved well at the ratio of 6:3:1(hexane, ethyl acetate and acetone) while ethanol extract were separated well at the ratio of 5:5 (hexane, ethyl acetate) (Table 8).

Table 2. Total phenolic content of different extracts

S. No	Type of extract	Total phenolic content (mg GAE/g sample)
1	Hexane	54±0.34
2	ethyl acetate	69.12±0.09
3	ethanol	62.1±0.42
4	Aqueous extract	47±0.01

Table 3. DPPH free radical activity of different extracts

S. No	Concentration (µg/ml)	Hexane	Ethyl acetate	Ethanol	Aqueous extract
1	10	16.23	14.06	18.68	17.30
2	25	27.31	26.95	24.75	26.50
3	50	34.61	40.10	31.43	38.79
4	100	49.47	54.22	39.02	44.92

Table 4. Reducing ability of the extracts

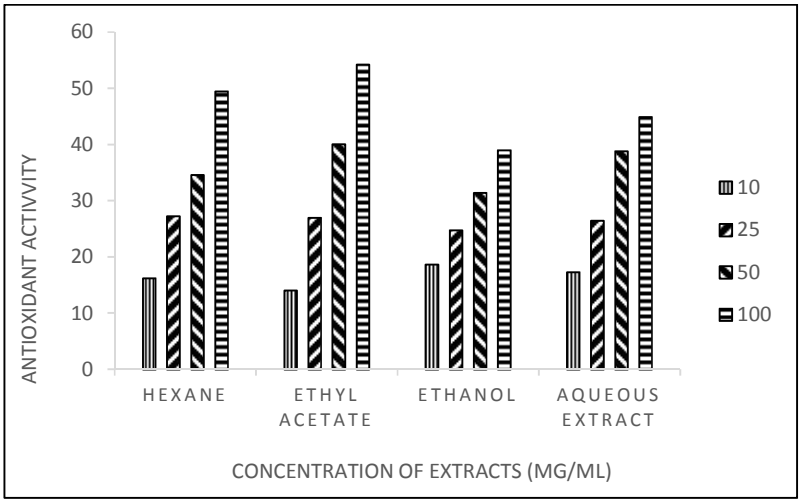
S. No	Concentration (µg/ml)	Hexane	Ethyl acetate	Ethanol	Aqueous extract
1	10	53.49	49.06	51.42	45.96
2	25	69.04	57.91	59.00	55.08
3	50	77.75	71.04	72.36	58.90
4	100	95.28	77.62	81.69	67.63

Table 5. Hydrogen peroxide activity of extracts

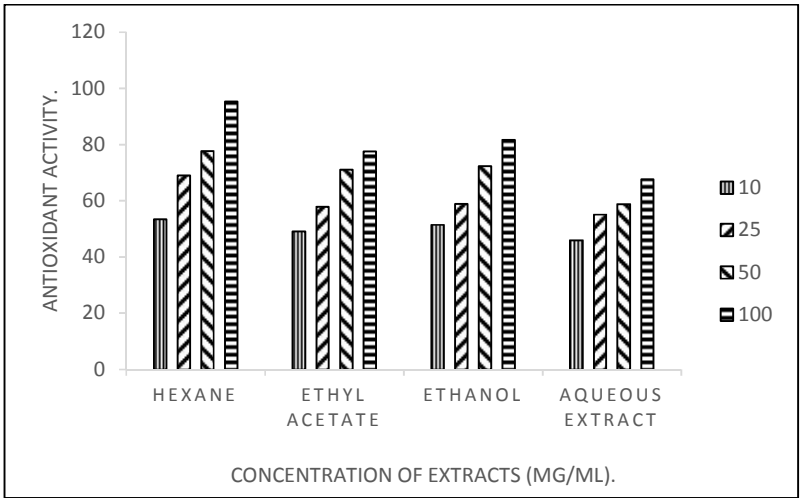
S. No	Concentration (µg/ml)	Hexane	Ethyl acetate	Ethanol	Aqueous extract
1	10	62.73	31.90	41.61	24.53
2	25	74.13	46.33	53.69	39.04
3	50	81.41	52.74	79.88	52.36
4	100	94.65	71.22	80.63	70.11

Table 6. Anti-fibrin activity of extracts at 25°C

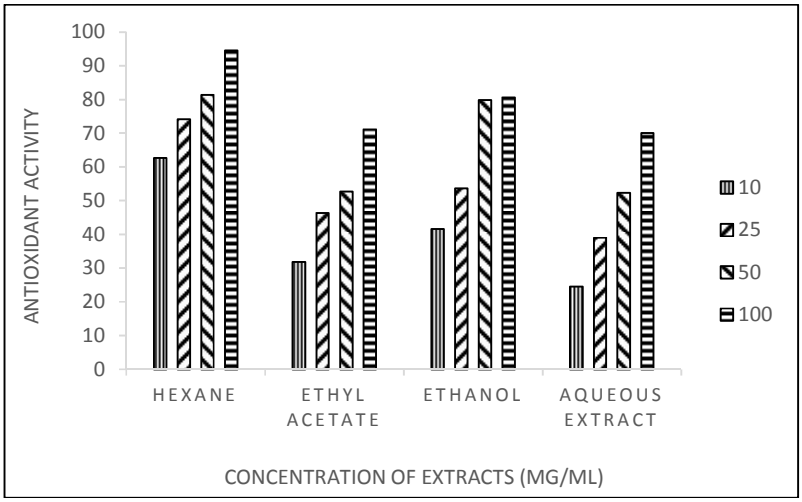
S. No	Initial clot (mg)	Type of extract used (50 µl)	Final clot (mg)	Percentage(%) lysis at 25°C
1	403	---	---	---
2	410	Hexane	385	6.09
3	406	Ethyl acetate	317	21.92
4	398	Ethanol	344	13.5
5	415	Aqueous extract	401	3.37
6	420	Distilled water	414	1.42



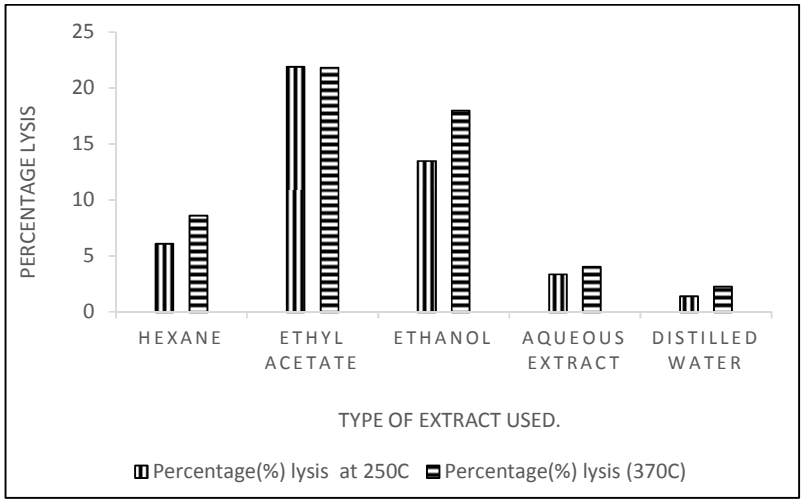
Graph 1. DPPH free radical activity of different extracts



Graph 2. Reducing ability of the extracts



Graph 3. Hydrogen peroxide activity of extracts



Graph 4. Comparison of anti-fibrin activity of extracts

Table 7. Anti-fibrin activity of extracts at 37°C

S. No	Initial clot (mg)	Type of extract used (50µl)	Final clot (mg)	Percentage(%) lysis (37°C)
1	400	---	---	---
2	406	Hexane	371	8.62
3	412	Ethyl acetate	322	21.84
4	411	Ethanol	337	18.0
5	420	Aqueous extract	403	4.04
6	396	Distilled water	387	2.27

Table 8. Separation of extracts by TLC

Extract	Separating solvent (hexane, ethyl acetate and acetone)	Number of spots
Aqueous	5:5:0	04
	6:4:0	04
	2:8:0	03
	6:3:1	04
	5:5:0	06
Hexane	6:4:0	07
	2:8:0	05
	6:3:1	11
	5:5:0	05
Ethyl	6:4:0	08
Acetate	2:8:0	08
	6:3:1	12
	5:5:0	10
Ethanol	6:4:0	09
	2:8:0	06
	6:3:1	07

4. CONCLUSION

Traditional medicinal system involves the use of numerous therapeutic measures for different diseases. Researchers already know the medicinal values of plants and presently investigating on the relation between the clinical metabolism and Medicinal values .Based on our study, it can be concluded that the leaves of *Piper betel.* L are potent sources of anti-microbial, anti-oxidant and anti-fibrin activities. The leaf extracts of *Piper betel. L* shows significant antibacterial effect due to the presence of many potent compounds such as alkaloids, tannins, phenolic substances and glycosides etc. *Piper betel* L was also tested for antioxidant property based on the fact that natural plant sources can scavenge free radicals generated in the body which would be helpful in curing many ailments related to human health. Future prospects involve exploring more about this herb for its potential in treating various diseases.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Sanghyun Lee, Dong-Sun Shin, Ju Sun Kim, Ki- Bong Oh, Sam Sik Kang. Antibacterial Coumarins from *Angelica gigas* roots. Archives of Pharmacal Research. 2003;26(6):449.
2. Santhakumari P, Prakasam A, Puglendi KV. Modulation of oxidative stress parameters by treatment with *Piper betel* leaf in streptozotocin induced diabetic rats. Indian Journal of Pharmacology. 2003;35: 373-8.
3. Lei D. Antioxidant and antiplatelet effect of aqueous inflorescence *Piper betel* extract. Journal of Agricultural Food and Chemistry. 2003;51:2083-8.
4. Majumdar B, Chaudhuri SR, Roy A. Potent antiulcerogenic activity of ethanol extract of leaf of *Piper betel* Linn. By antioxidative mechanism. Indian Journal of Clinical Biological Chemistry. 2002;17:49-57.
5. Adhikari P, Chowdhury D, Baneerji J, Chatterjee A. Antifertility effect of crude alcoholic extract of *Piper betel* stalk. Indian Journal of Physiology and Allied Sciences. 1998;52:22-7.
6. Adhikary P, Banerji J, Choundhuri D, Das AK, Deb CC, Mukherjee P, Chatterjee SR. Effect of oral administration of stalk of leaves of *Piper betel* Linn on oestrous cycle and its antifertility activity in rats. Indian Journal of Physiology and Allied Sciences. 1990;44:116-23.
7. Chen SJ, Wo BN, Yeh JL, Lo YC, Chen IS, Chen IJ. C-fiber evoked autonomic cardiovascular effect of after injection of *Piper betel* inflorescence extracts. Journal of Ethno Pharmacology. 1995;43:183-8.
8. Bhide SV, Zariwala MB, Amonkar AJ, Azuine MA. Chemo preventive efficacy of a *Piper betel* leaf extract against benzo (a) pyrene induced for stomach tumors in mice. Journal of Ethno Pharmacology. 1991;34:207-13.
9. Amonkar AJ, Padma PR, Bhide S. Protective effect of hydroxychavicol, a phenolic component of *Piper betel* leaf against the tobacco-specific carcinogens. Mutation Research. Fundamental and Molecular Mechanisms of Mutagenesis. 1989;210:249-53.
10. Varier PS. *Piper betel* Linn. In: Varier PS. (ed) Indian medicinal plants. Kottakkal: Oreint Longman. 1997;8:279-283.
11. Koff RS, Gordan G, Sabesin SM. D-galactosamine hepatitis hepatocellular injury and fatty liver following a single dose. Proceedings in Society of Experimental Biology and Medicine. 1971;137:696-701.
12. Shun CY, Chau JW, Jing JL, Pei LP, Jui LH. Fen PC. Protection effect of *Piper betel* leaf extract against carbon tetrachloride induced liver fibrosis in rats. Archives of Toxicology. 2007;81:45-55.
13. Maisuthisakul P, Pongsawatmanit R, Gordon MH. Assessment of phenolic content and free-radical scavenging capacity of some thai indigenous plants. Food Chemistry. 2007a;100:1409-1418.
14. Marxen K, Vanselow KH, Lippemeier S, Hintze R, Ruser A, Hansen UP. Determination of DPPH radical oxidation caused by methanolic extracts of some microalgal species by linear regression Analysis of Spectrophotometric Measurements, ISSN1424-8220, 2007.
15. Aiyegoro OA, Okoh AI. Preliminary phytochemical screening and in vitro antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC. BMC Complementary and Alternative Medicine. 2010;10(21):1-8.
16. Priyanka Chakraborty, Pallab Dasgupta, Bala NN. Comparative study of *in vitro* antioxidant activity of different extracts of piper betel leaves, ISSN 2229 – 6859. 2013;3(13).
17. Nair R, Chandra S. Antimicrobial activity of Terminalia, Manikkara Zapota and *Piper betel* Leaf Extract. Indian J Pharm Sci. 2008;70(3):390-393.
18. Shitut S, Pandit V, Mehta BK. The antimicrobial efficiency of *Piper betle linn*

leaf (stalk) against human pathogenic bacteria and phytopathogenic fungi. Cent Eur J Public Health. 1999;7(3):137-9.

19. M Mahfuzul Hoque, Shemona Rattila, M Asaduzzaman Shishir, M L Bari, Y Inatsu, S Kawamoto. Antibacterial activity of ethanol extract of betel leaf (*Piper betle* L.) against some food brone pathogens. Bangladesh Journal of Microbiology. 2011;28(2):58-63.

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