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Characterization and evaluation of in vitro anticancer, antioxidant and antimicrobial activity of methanolic extract of Piper betle

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Abstract : The leaves of Piper betle Linn. have long been used in Ayurveda and alternative system of medicine. They are considered auspicious and are still extensively used during religious functions. Betel leaf is consumed as a digestive stimulant and mouth freshener. It is said to have a number of medicinal properties, including anti-inflammatory, analgesic, antipyretic and anti-oxidant activities. In our present study, we evaluated the anticancer, antioxidant and antimicrobial activities of methanolic extract of Piper betle. The phytochemical screening of crude methanolic extract of betel leaves revealed the presence of phytochemicals like Alkaloids, Carbohydrates, Tannins, Glycosides, Steroids, Saponins, Flavanoids and Volatile oils, while Glycoproteins were found to be absent. HPLC and Gas Chromatography analysis was undertaken to find out the bioactive compounds, which showed major peaks pertaining to Chromanol, 4-Acyl 1,2 diacetoxy benzene and Phenol 2-methoxy-4-(2-Propenyl acetate). MTT assay followed by DNA fragmentation test and apoptosis using HCT-116 cell line indicated significant cytotoxic activity by the extract. It also showed statistically significant antioxidant activity as evidenced through DPPH activity and lipid peroxidation assay. Antimicrobial activity was evaluated using well-diffusion method, and Piper betle showed effective antibacterial activity against B. cereus, E. coli, P. aeruginosa and S. typhi.

Keywords: Piper betle, Anticancer, DPPH, Lipid peroxidation, Antimicrobial activity.

1. INTRODUCTION

According to the World Health Organization (2009), 7.9 million people worldwide died of cancer in 2007. This number is projected to increase to 12.0 million in 2030. Cancers may be caused in one of three ways, namely incorrect diet, genetic predisposition, and via the environment. At least 35% of all cancers worldwide are caused by an incorrect diet, and in the case of colon cancer, diet may account for 80% of the cases. According to the national cancer registry program of the Indian Council of Medical Research, more than 1300 Indians die every day due to cancer. In India, the annual incidence rates for colorectal cancer and rectal cancer in men and women are 4.4 and 3.9 per 100000 respectively. In our study we selected the leaves of the plant Piper betle to evaluate its in vitro anticancer activity.

The leaves of Piper betle Linn. have long been use in the Indian local system of medicine. Piper betel Linn. belongs to family Piperaceae and commonly known as the betel vine, which is an important medicinal and recreational plant in Southeast Asia [1]. Betel leaves are consumed after heavy meals as a digestive stimulant and mouth freshener. Paan is said to have a number of medicinal properties, including anti-inflammatory and anti-oxidant activities [2]. It has phytochemical components like alkaloids, flavonoids, triterpenoids, anthracene glycosides, tannins, phenolics and saponins by phytochemical assay which has antioxidant and antibacterial properties [3]. Piper betle also contains important chemical constituents which are known for its medicinal properties like anti-allergic, anti-malaria, anti-filarial, antibacterial, antifungal study, insecticidal, antioxidant, anti-diabetic, gastro-protective, cyto-toxic, anti-platelet, wound healing activity [4]. Hence, betal leaves can be used in the preparation of medicines due to its reported antioxidant and antimicrobial properties [5]. This study has been undertaken to evaluate the biological activities, anticancerous, antioxidant and antimicrobial activity, of Piper betle.

2. MATERIALS AND METHODS

2.1. Prepration of leaf extract

The healthy Piper betle leaves were collected and washed thoroughly with running tap water and shade dried. 20g of dried sample powder was dissolved in 100 ml of methanol in a 500ml beaker with aluminium foil covered on it. The beaker was kept on hot water bath at 50º C for 4 hours. After incubation period the extract was filtered with Whatmann filter paper and the filtrate was collected in a 50 ml beaker and the filtrate was taken for further use. The filtrate was

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kept at 50ºC for few hours until the extract completely dried and turned into a semisolid form. This semi solid sample was weighed and the yield was noted and stored for futher use.

2.2. Phytochemical analysis

Phytochemical analysis of the selected plant was done using standard chemical test [6]. The tests were carried out in five replicates by using uniform concentration of the samples. After extraction, phytochemical screening was done for alkaloids, carbohydrates, tannins, tannins, terpenoids, glycosides, steroids, saponins, flavonoids, mucilage test, volatile oil and phenols.

2.3 Cytotoxicity studies for HCT 116 cell line

Cytotoxicity assay was done by using modified method of Crouch [7]. Studies were carried out at Skanda life science Bangalore. The tests were carried out in five replicates by using uniform concentration of the samples.

% Inhibition = (OD of Control – OD of sample)/OD of Control) x 100.

2.4Apoptotic study by flow cytometry

Apoptosis was studied following the Flow cytometry method of Koopman [8]. Studies were carried out at Skanda life science Bangalore.

2.5 Antioxidant (DPPH scavenging assay)

Antioxidant assay was done following the DPPH method of Kasapoglu [9]. Studies were carried out at Skanda life science Bangalore.

2.6 Lipid peroxidation assay

A modified thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenate as lipid rich medium.

2.7 Antimicrobial activity

The antimicrobial assay was done by agar well diffusion method [11]. Studies were carried out at Skanda life science Bangalore. The tests were carried out in five replicates by using uniform concentration of the samples.

Test organisms: Bacillus cereus, Escherichia coli, Psuedomonas aeruginosa and Salmonella typhimurium..

Positive control: The test compound used as standard was Ciprofloxacin (0.1mg/ml).

The treated plates were observed for zone of inhibition around the wells.

3. RESULTS:

Table 1: Phytochemical constituents in crude methanolic extract of Piper betle.

Types of Tests	Result
Alkaloid	+
Carbohydrate	+
Tannin	+
Terpenoid	+
Glycoside	+
Steroid	+
Saponin	+
Flavanoid	+
Proteins (Myllon's Test)	+
Glycoprotein Test	-
Volatile Oil	+

+ve : indicates the presence of compounds
-ve : indicates the absence of compounds

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3.1 Anticancer Activity

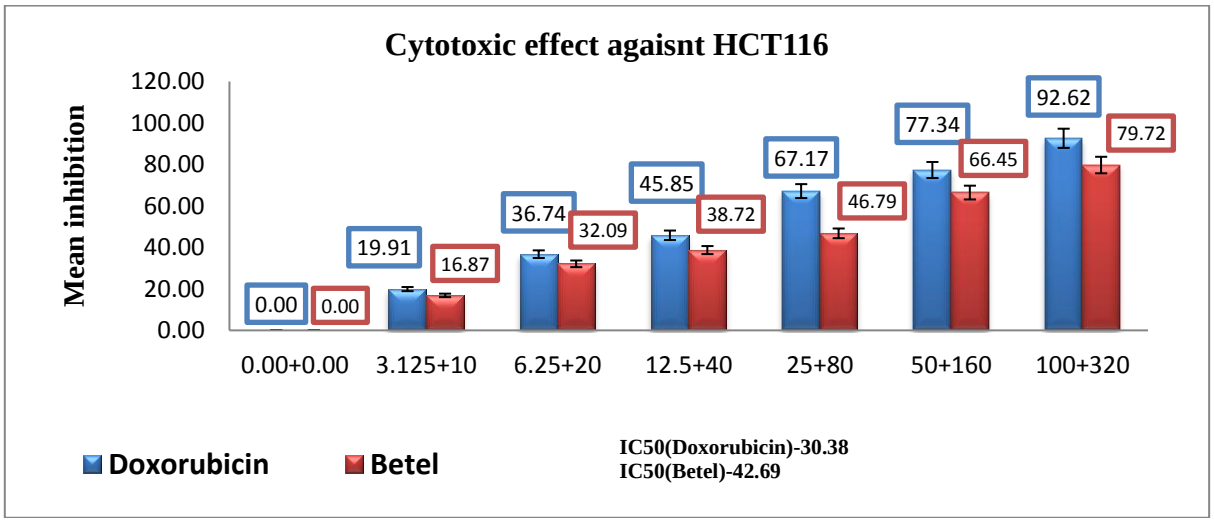

Figure 3.1: Cytotoxic effect of various concentrations of Piper betle against HCT-116.

3.2 Flow Cytometry

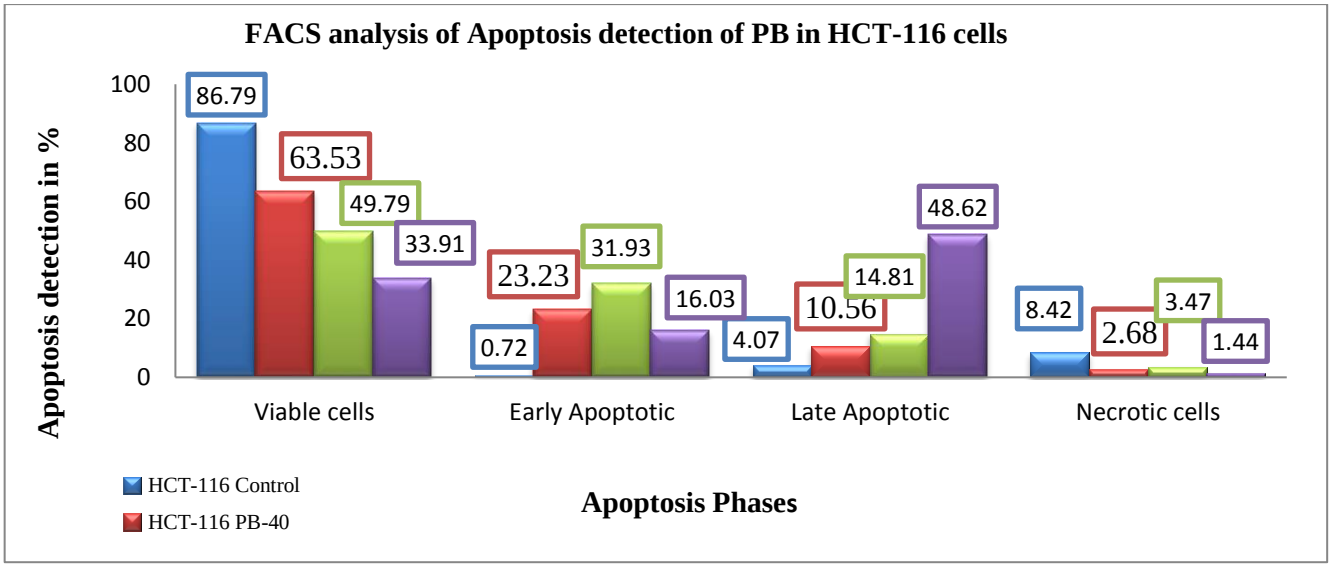

Figure 3.2: Graph showing the viable cells, early apoptosis, late apoptosis and necrotic cells detection of PB in HCT-116cells.

In flow cytometry analysis for apoptosis detection of PB in HCT-116 cells, two concentrations of the sample were taken, viz., 40µg/ml and 80µg/ml. In this analysis the peak significantly increased in treated cells compared to control. It was found that with increase in the concentration of the extract the number of viable cell decreased, whereas the early and late apoptotic cells increase. The number of necrotic cells also showed an increase with increase in the concentration of the extract. This indicates that Piper betle has apoptotic activity.

3.3 Anti-oxidant Activity (DPPH assay)

Table 2: DPPH activity of methanolic extract of Piper betle.

Sample*	Concentration (µg/ml)	Mean inhibition ± SEM
Control	0	0±0

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Quercetin	0.3125	13.89± 0.4651
	0.625	24.25±0.1825
	1.25	37.86±0.8513
	2.5	48.61±0.6961
	50	62.33±0.7722
	100	78.82±0.7647
Control	0	0±0
Piper betle	12.5	14.10± 0.39
	25	20.39±0.61
	50	36.45±0.59
	100	54.58±0.50
	200	62.34±0.43
	400	79.64±0.73

3.4 Lipid Peroxidation Assay

Table 3: TBARS assay of Piper betle.

Sample*	Concentration (µg/ml)	Mean inhibition ± SEM
Control	0	0.00±0.00
MDA Standard	0.063	0.0318±0.0037
	0.125	0.059±0.00070
	0.25	0.114±0.00074
	0.5	0.114±0.00081
	1	0.23±0.00086
	2	0.452±0.00067
Control	0	0.00±0.00
Piper betle	0.625	11.24 ±1.05
	1.25	29.23±0.84
	2.5	39.62±0.29
	5	55.27±0.37
	10	64.15±0.51
	20	80.99±0.77

*Five replicates.

3.5 Anti-bacterial Activity (By Well Diffusion Method)

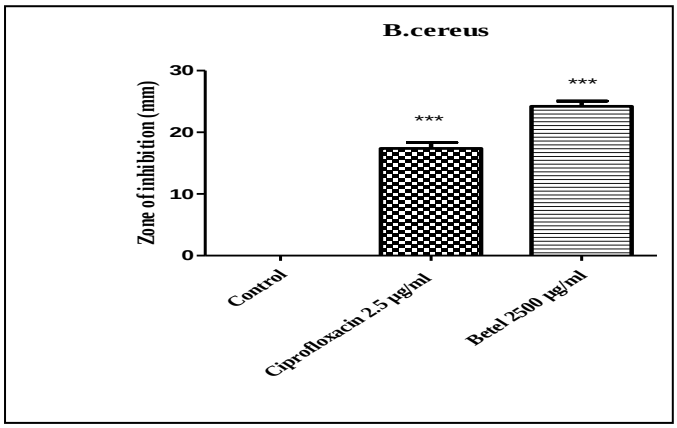

Figure 3.3: Antibacterial activity of methanolic extract of *piper betle* and standard Ciprofloxacin against *B.cereus*.

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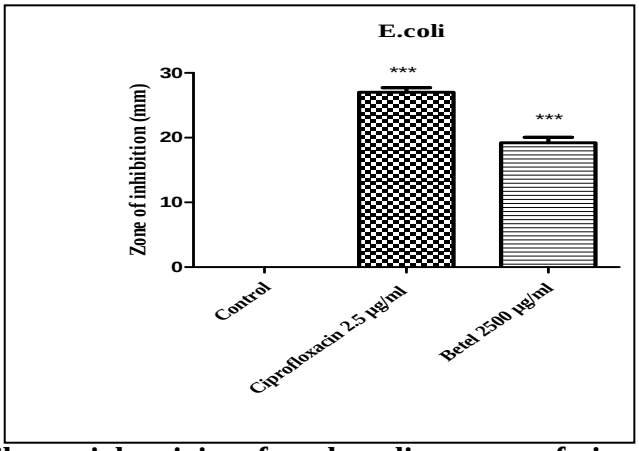

Figure 3.4:Antibacterial activity of methanolic extract of *piper betle* and standard Ciprofloxacin against, *E.coli*.

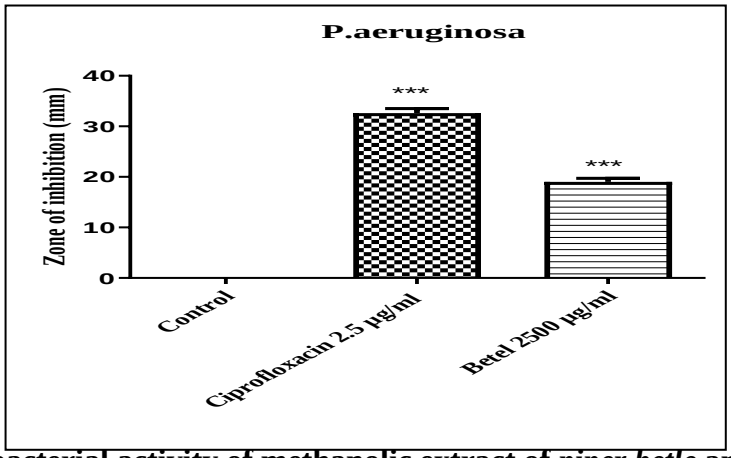

Figure.3.5: Antibacterial activity of methanolic extract of *piper betle* and standard Ciprofloxacin against *P.aeruginosa*.

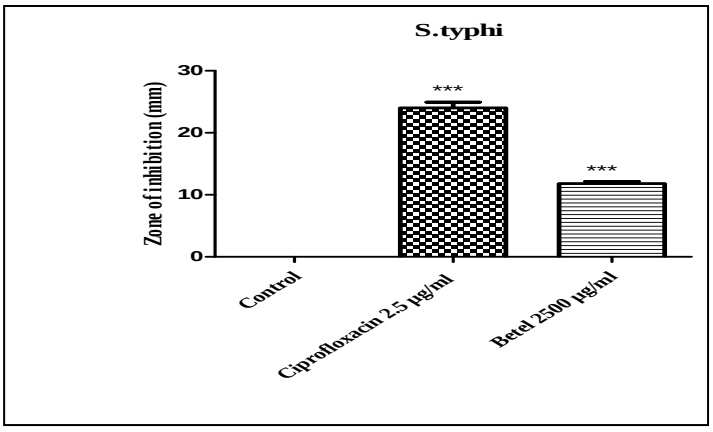

Figure 3.6: Antibacterial activity of methanolic extract of *piper betle* and standard Ciprofloxacin against *S.typhi.*

DISCUSSION

Many plant species are already being used to treat or prevent development of cancer. Multiple researchers have identified species of plants that have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries [12]. To increase the sustainability of medicinal plants in developing

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countries, utilization of all plant parts including the stem, leaf, root and bark should be included in the treatment [13]. Cancer has been a constant battle globally with a lot of development in cures and preventative therapies. The disease is characterized by cells in the human body continually multiplying with the inability to be controlled or stopped [14].

In our study, Piper betle leaf extract was used against HTC-116 cell line i,e colon cancer cell line for the treatment of cancer. Betel leaf extract showed effective results with reference to cytotoxic and apoptotic effects on colon cancer cells. Betel extract also showed effective zone of inhibition against gram positive and gram negative bacteria. P.betel should certainly find place in the treatment of various bacterial infections and cancer treatments. From the results obtained it is evident that the selected plants which is well known for its medicinal properties contain almost all the prominent phytochemicals. The combination of these secondary metabolites in the plants consumed as food/ medicine provide the synergistic effect for prevention and treatment of various diseases. Value addition may be by means of food product development or medicinal product development [15]. Phytochemical studies show that Piper betle contains a wide variety of biologically active compounds. The aroma of betel leaf is due to the presence of essential oils, consisting of phenols and terpenes [16]. In the earlier study different medicinal compounds such as glycosides, carbohydrates, amino acids, Saponins, flavones, tannins, phenolds, oils and fat were present and alkaloids, steroids, glycosides were absent in betel [17]. This property makes it to be fit for its future usage as a promising source for treating various conditions. The same with lots of biological activities has a tremendous strength to come out as a future herbal medicine and nutritive value [18].

HPLC analysis was carried out on Piper betle of methanolic extract. Among all seven peaks, retention time 2.790 showed maximum area of 66.5 %. The identification tests are required to confirm the presence of the active constituents and potential adulterant in ayurvedic drugs. These peaks showed that there is presence of different compounds. HPLC analysis on aqueous and ethanol extracts from P. betle revealed the presence of hydroxychavicol (4-allyl pyrocatechol) (12) (Rt: 18.5 min; UV: 283 nm), as the main compound . More compounds were detected in the aqueous extract than in the ethanol. [19]. GCMS analysis was undertaken to find out the bioactive compounds present in Piper betle. The compounds were identified by comparing their retention time and covate indexes with that of literature and by interpretation of mass spectra [20].

In the Anti-cancer study, we utilized colon cancer cell line, i,e., HCT-116 with specific characterstics, to test the cytotoxicity and to know whether these cancer cells were resistant to the treatment of PB leaf extract. Traditionally, the in vitro determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of effects caused by the test material [21]. Piper betle leaves have potent anticancer properties due to the presence of phytochemicals, free radical activity as well as inducing selective toxicity against cancerous cells [22]. The Cytotoxic effect of ethanolic extract of Piper betle was determined on four different cancer cell lines, two murine (Ehrlich Ascites carcinoma and Melanoma B-16 cells), and two human (HeLa and Raji cells). Where the Piper betle, cytotoxic effect on tumor cells was greater than that on normal cells [23].

The studies conducted on fruits, seeds, leaves, and plant roots were used for in vitro and in vivo studies to provide an overview of medicinal plants was effective on colon cancer with special emphasis on bioactive components and underlying mechanisms of action [24]. Piper betel, a treasure of bioactive phenolics, possesses great potential to fight against cancers of oral, mammary, prostate, skin, and gastric origin. Much of these powers of betel leaf phytochemicals remain unharnessed and call for extensive research to better understand their mechanisms of action and clearly demarcate their chemopreventive and chemotherapeutic roles [25].

HCT-116 cells are used in a variety of biomedical studies involving colon cancer proliferation and corresponding inhibitors. The cell line has been used in tumorigenicity studies, along with other research that has shown that cyclin D1 holds large importance for the activity of lithocholic acid hydroxyamide [26]. Studies were carried out on anticancer activity of the nanobioconjugates synthesized from the betel leaf extract and pure compound, in comparison with their respective non-conjugated raw material using lung cancer. This method was used to evaluate the effect of silver nanobioconjugates on the viability of A549 lung cancer cells in comparison with non-cancerous peripheral blood lymphocytes. It is reported that P. betle leaves and their major polyphenol (EU) possess strong anticancer activity in the nanoform. By using an inexpensive and eco-friendly sunlight exposure method, the nanobioconjugates could be synthesized from the betel plants, which is enriched with the medicinal properties and easily available and medicinal rich [27].

The studies were carried out on apoptosis using HTC-116 cell line, on Piper betle Methanolic extract. By conjugating FITC to annexin V it is possible to identify and quantitate apoptotic cells on a single cell basis by flow cytometry. In flow cytometry analysis for apoptosis detection of PB in HCT-116 cells, two concentrations of the sample were taken, viz., 40µg/ml and 80µg/ml. In this analysis the peak significantly increased in treated cells compared to control. It was found that with increase in the concentration of the extract the number of viable cell decreased, whereas the early and late apoptotic cells increase. The number of necrotic cells also showed an increase with increase in the concentration of the extract. This indicates that Piper betle has apoptotic activity. It was reported that, Hydroxychavicol treated cells

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exhibited condensation of DNA revealed by Hoechst staining quantified by flow cytometry. [28]. The intrinsic higher oxidative stress in cancer cells might be exploited to preferentially kill these cells in vitro by agents that induce intracellular ROS (reactive oxygen species) accumulation [29].

Studies were conducted by us on Anti-oxidant activity of Piper betle leaf by DPPH assay. Studies were carried out with Standard Quercetin and sample Piper betle methanolic extract. Free radical scavenging activity of Piper betle was seen at higher concentration and inhibition of 79.64% was observed. Similarly, Quercetin at higher concentration showed inhibition of 78.82%. Hence the free radical scavenging activity of both sample and standard were almost similar at higher doses. DPPH radical scavenging activity gives an idea of Anti-oxidant activity of the compound, and is expressed as the concentration of sample needed for 50% reduction of DPPH colour intensity.

The identification of antioxidants may reduce the risk of various chronic diseases involved in free radicals. The upshots of oxidative stress are serious and sometimes manifested by increased activities of enzymes involved in oxygen detoxification [30]. Studies were conducted on six varieties of betel leaves and it was reported that varying polarities of extraction solvent proved to be an effective tool in extraction of bio-actives compounds. Addition of 20% water in methanol, ethanol, acetone, ethyl acetate can improve the performance of extracting solvent and extract more antioxidant compound from the betel leaves which could be the promising source of natural antioxidant for food and pharmaceutical industries [31].

Studies were carried out on Red betel using 70% ethanol extract active compounds that have antioxidant activity, such as flavonoids, polyphenols, alkaloids, and tannins andit was reported that the red betel leaf extract (P. crocatum Ruiz) and its compounds (eugenol and hydroxychavicol) have antioxidant activity as indicated by the results of the DPPH scavenging test [32]. It was reported that the P.betle leaf extract showed less antioxidant activity in Nitric oxide and hydroxyl radical assays compared to eugenol, whereas in reducing power assay, P. betle leaf extract showed good antioxidant activity [33].

We evaluated Lipid peroxidation activity of Piper betle methanolic extract and MDA standard and it was found that lipid peroxidation activity was seen at higher dose compared to MDA standard. It was reported that TBARS levels in the cancerous tissues was higher than those in the normal tissues [34]. The increase in lipid peroxidation showed at higher doses of betel leaf extract may be due to fall in total radical trapping capacity of blood plasma and marked reduction in plasma levels of antioxidants [35].

The studies conducted on Antibacterial activity of methanolic extract of Piper betle leaves are explained in Table 8. All the selected test organisms showed significant zone of inhibition. B.cereus showed higher inhibition (24.2) compared to Ciprofloxacin (17.4). While other organisms like E.coli, P. aeruginosa and S.typhi showed lesser inhibition compared to Ciprofloxacin. The significant antibacterial effect may due to the presence of many potent compounds such as alkaloids, tannins, phenolic substances and glycosides etc. The results of this study indicate that this herb should be studied more extensively to explore its potential in the treatment of many infectious diseases.It is also reported that due to the presence of phenolic compounds, crude extract of P.betle leaves showed significant antimicrobial activity [36]. Antibacterial activity of crude aqueous extract of Piper betle showed activity against most of the test bacteria, with the greatest zone of inhibition by the ethanol extract against Gram negative and Gram positive bacteria, with maximum bactericidal activity against E. coli, P.aeruginosa, and S.aureus. Thus betel leaf can be used as antimicrobial agent [37]. It was observed that alcohol and water extracts possess antibacterial activity against different gram positive and gram negative bacteria showing that it can be used as a natural antimicrobial agent [38]. Higher concentrations of crude extracts show more zone of inhibition towards gram positive bacterial strain, whereas lower concentration of crude drug extract show more zone of inhibition towards gram negative strains [39].

CONCLUSION

In our study, Piper betle leaf extract was used against HTC-116 cell line i, e colon cancer cell line for the treatment of cancer. Betel leaf extract showed effective results with reference to the cytotoxic and apoptotic effects on colon cancer cells. Betel extract also showed effective zone of inhibition against gram positive and gram negative bacteria. P.betel should certainly find place in the treatment of various bacterial infections and cancer treatments. Hence, medicinal plants play an important role in providing primary health care. The results of this study were very encouraging and indicate that this herb should be studied more extensively to explore its potential in the treatment of many infectious diseases.

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