Phytochemical Investigation and Biological Evaluation of *Erythrina Suberosa* Bark Extract

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ABSTRACT

Plants are considered great and indispensable source of food, energy, cosmetics and even medicines. Since prehistoric culture, humans rely on plants for their growth, survival and still cultivated vegetables and plants as a source of novel chemical compounds with pharmacological properties. Current study was designed to separate active constituents of *Erythrina suberosa* bark extract through phytochemical screening and evaluated their biological activities such as anticancer, anti-inflammatory and antioxidant activities. The bark extract of *E. suberosa* contained saponins, glycosides, alkaloids, tannins, terpenoids and phenols, identified by phytochemical analysis. Furthermore, the bark extract shows the concentration dependent cytotoxicity against HT-144 cell lines evaluated through MTT assay. In addition, the *E. suberosa* bark extract also exhibited anti-inflammatory effect at higher doses (400 mg/kg) and moderate antioxidant activity is noticed through DPPH free radical assay. These findings indicate that *E. suberosa* bark extract exhibited prominent anticancer and anti-inflammatory activity and might be serve as a potent therapeutic agent in future studies.

Key words: *Erythrina suberosa*, bark extract, phytochemicals, HT-144 cell line, anticancer, anti-inflammatory.

1. Introduction

Medicinal plants still have been applied for different diseases worldwide in different human civilizations. Herbal medicines are considered to be more affordable, easily available, safer and free of toxic side effects disparate to their synthetic counterparts. Approximately 3.3 billion peoples of developing countries utilize medicinal plants for the treatment of different diseases [1]. Medicinal plants are considered as an abundant source of active compound applied in drug development and synthesis. According to WHO bulletin, traditional herbal medicines and phytonutrients or nutraceuticals are getting significant devotion in health debates globally [2]. In Africa, about 80% of population uses some form of herbal medicine and the annual market for herbal products reaches to about US$ 60 billion. In Canada, France, and India 50%, 75%, and 65% of the population, respectively, depends on herbal medicine alone or in combination with orthodox pharmaceuticals. Furthermore, 85% of Japanese doctors prescribe traditional herbal medicine, instead of modern medicine [3]. In China, herbal medicine played a bulging role in the treatment strategy of severe acute respiratory syndrome (SARS). Another study shown that about 40% of all healthcare services provided in China depends on herbal medicine, while the herbal medicine dependent population of developed countries (Canada, Australia, USA, and Belgium) is estimated between 38% - 75% [4, 5]. *Erythrina suberosa* is a medium size (10 m) deciduous tree commonly known as corky coral Indian coral tree, belongs to pea family and widely used for ornamental purposes in Pakistan (Fig.1). *E. suberosa* is found to be rich in secondary metabolites, particularly flavonoids, phenolics and alkaloids that exhibit promising anti-inflammatory, antiplasmodial, bactericidal and fungicidal activities. Alkaloids, obtained from *E. suberosa* flowers, were investigated for anxiolytic activities, and the obtained results displayed significant anxiolytic effects *in-vivo* [6]. Erythraline alkaloid is nicotinic receptors α-4/β-2-antagonist, and exhibited protruding anxiolytic effect [7]. Further, the crude aqueous extract of *E. suberosa* flowers showed potential bronchodilatory, spasmolytic, and antioxidant properties [8]. From the aqueous methanolic extract of *E. suberosa* of stem bark, certain alkaloids and saponins were isolated, which have

exhibited prominent antibacterial, antifungal and cytotoxic effects against certain strains of human pathogens [9]. Current study was design to identify the active constituents of *E. suberosa* bark extract by phytochemical screening and further explored it's an anti-inflammatory, anticancer and antioxidant properties *in-vitro* and *in-vivo*.

2. Material and Methods

2.1 Plant Collection

The bark of *E. suberosa* (Roxb) plants were carefully collected from Islamabad, Pakistan during summer season (april 2019) and were recognized by an expert taxonomist (Dr. G.A. Miana), department of pharmaceutical chemistry, Riphah International University, Islamabad, Pakistan, where a voucher sample was placed for reference purpose.

2.2 Crude Extract Preparation

The collected plant materials were carefully rinsed with water in order to remove all adulterated foreign particles followed by layer spreading on paper sheet and subjected to shade drying. These plant materials were protected from direct and intense sunlight exposure to minimize the chances of losing active constituents. The dried bark was the cut into small pieces using a specified electric cutter. About 1.5 kg of dried bark pieces were placed in a percolator and soaked in 2 liters of chloroform for two weeks at room temperature. Subsequently, the extract was collected in 1000 ml beaker were soaked in 2 liters of chloroform at room temperature with occasional shaking. The mixture was passed through muslin cloth followed by the subsequent filtration through grade no. 1 Whatman filter paper. The filtrate was further concentrated using rotary evaporator at 40 $^{\circ}$C under reduced pressure with final yield of about 8%. The obtained concentrated extract was stored at 4 °C for future use. The extract was dissolved in sterilized water prior to experiments to obtain the stock solution of extract, which was further diluted accordingly for investigation purposes [10].

2.3 Phytochemical screening of *E. suberosa* bark extract

The crude extract of *E. suberosa* bark were analysed for the different phytochemicals, such as saponins, glycosides, alkaloids, tannins, terpinoids and phenols, by using different reagents as mentioned [11-15].

2.3.1 Tests for saponins

Briefly, *E. suberosa* bark extract was placed in 2 ml tube and subsequently a small quantity lead acetate was added dropwise and observe extract solution for any change.

2.3.2 Test for cardiac glycosides

Crude extract of *E. suberosa* bark was taken in a test tube and treated with glacial acetic acid (2 mL). Further, 1 mL $FeCl_3$ and 1 mL of concentration sulphuric acid was added to the test tubes, respectively. Observe the formation of reddish black layer for the presence of cardiac glycosides.

2.3.3 Tests for alkaloids

E. suberosa bark extract solution (1 ml) was acidified with acetic acid solution (10%) in two separate test tubes A & B. To test tube A few drops of Mayer's reagent were added, whereas to the test tube B few drops of freshly prepared Dragendorff's reagent were added. The appearance of cream-colored precipitates and reddish-brown precipitate with with Mayer's reagent and Dragendorff's reagent, respectively confirmed the presence of alkaloids.

2.3.4 Tests for tannins

Few drops of ferric chloride ($FeCl_3$, 5%) solution were gently added to crude extract of *E. suberosa* bark, resulting in bluish black colour, which confirmed the presence of tannins.

2.3.5 Test for terpenoids

Crude extract of *E. suberosa* bark was taken in test tubes (5 mL); 3 mL chloroform was added, to the test tube followed by addition of 3 mL concentrated sulphuric acid (H_2SO_4) carefully. The presence of terpenoids was confirmed by the reddish-brown colour of the interface.

2.3.6 Test for flavonoids

Crude extract of *E. suberosa* bark was taken in test tubes and then 5 mL of dilute ammonia solution was added to the aqueous filtrate of extract. Subsequently, concentrated H_2SO_4 was added carefully. Appearance of yellow colour was observed for flavonoids.

2.3.7 Test for phenols

Crude extract of E. *suberosa bark* was taken in test tubes, followed by dropwise addition of 5% $FeCl_3$ into test tube, and observed for bluish black colour of phenols [16].

2.4 Characterization of *E. suberosa* Bark Extract

Fourier transform infrared (FTIR) spectrum of extract was measured by directly placing the sample on the crystal plate centre of FTIR spectroscope (Perkin Elmer, USA), and measured the spectrum in a range of wavenumber 500–4000 cm^{-1}. The resultant peaks were compared with the previous reported studies [17-20]. GCMS technique has been used for identification of phytochemicals. Phytochemicals constituents protect us from disease Phytochemical constituents of *E. suberosa* were measured by using standard procedure and the equipment used for this process [21].

2.5 Biological Evaluation of *E. suberosa* Bark Extract

2.5.1 Antioxidant activity

Briefly, 1 ml of DPPH solution (0.1 Mm, methanol) was mixed with 1 ml extract samples of varying concentration (0, 25, 50, 100, 200, 400 μg/mL). The reaction mixture was gently shaken and further incubated in dark for 1 hour at room temperature. After 1 hour incubation, the absorbance was measured at 517 nm wavelength by multifunctional microplate reader. Vitamin C (ascorbic acid) was used as standard antioxidant agent. The DPPH radical inhibition caused by the extract sample was calculated using the formula below:

$$\text{Scavenging Activity (\%)} = \frac{Absorbance\ of\ Standard - Absorbance\ of\ Sample}{Absorbance\ of\ Standard} \times 100$$

2.5.2 Anticancer activity

MTT assay was conducted to explore the cytotoxic effect of *E. suberosa* bark extract against human fibroblast malignant melanoma (HT-144) cell line [22]. Briefly, HT-144 (5×10^5 cells/well) were cultured with 100 µL growth medium in 96-well plates and incubated for 24 h to adhere. After 24 h incubation, discarded the culture medium, and cells were treated with *E. suberosa* bark extract at serial concentrations ranging from 0 - 400 mg/mL and further incubated for 24 h, in triplicates. The untreated cells was used as control. After treatment completion, cells were washed 3 times with PBS and supplied with fresh medium (100 µL) followed by the addition of 20 µL MTT solution (5 mg/mL), and incubated for 4 h. Next, the culture media was removed and 150 µL DMSO was supplied to each well and vibrated to make it fully dissolve the formazan crystals produced by the living cells. Cells without treatment were used as control. Finally, the absorbance value which corresponds to the living cells was measured at 492 nm using multiple microplate reader. The percent of cells viability was calculated as the ratio of absorbance of treated cells to untreated cells using following formula. The half maximum inhibitory concentration (IC_{50}) was calculated from the dose-response curve.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance value of experimental group}}{\text{Absorbance value of control group}} \times 100$$

2.5.3 Anti-inflammatory activity

The *in-vivo* anti-inflammatory activity of *E. suberosa* was determined through carrageenan induced hind paw edema by using rats. The rats were obtained from the animal house of pharmacology department of Riphah Institute of health sciences. Rates were divided into four groups, namely standard, control, diseased and experimental groups) and each group contain equal rats. 0.4% DMSO was given to Group I which served as control group. Group II was given with 1% carrageenan and it was diseased group. Group III was given 10 mg/kg diclofenac sodium intraperitoneally in hind paw of rats before carrageenan and this was standard group. Experimental group was further divided into three groups. Rats of group (a), (b), (c) were given with 100 mg/kg, 200 mg/kg and 400 mg/kg oral dose of test compound respectively for 5 days. On 5^{th} day of experiment carrageenan was given intradermally in hind paw after 30 mins of test compound administration. The plethysmometer was used to measure volume changes in hind paw edema before and 1.0, 3.0 and 5.0 hrs after the injection of carrageenan.

2.6 STATISTICAL ANALYSIS

The experiments were reproduced thrice independently to ensure the consistency of the data.

The values were expressed as the mean ± standard deviation (SD).

3. Results

3.1 Phytochemical analysis

Crude extract of *E. suberosa* bark was studied for phytochemical screening with different reagents to identify the respective active constituents. The identified constituents are depicted in table 1.

Active constituent	Test performed	Observation
Saponins	Lead acetate test	White precipitate
Glycoside	Keller-Kalian's test	Brown ring interface
Alkaloids	Mayer's reagent	Yellowish precipitate
	Dragendorff's reagent	Reddish brown precipitate
Tannins	$FeCl_3$ test	Black colour
Flavonoids	Shinoda 's test	Red pink colour
Phenols	$FeCl_3$ test	Bluish black colour

Table. 1 Phytochemical analysis of crude extract of Erythrina suberosa bark

3.2. Antioxidant activity

DPPH radical is a stable organic free radical with an absorption band at 517 nm. It loses this absorption when it accepts an electron or a free radical species, resulting in a visually noticeable discoloration from purple to yellow. It can incorporate many samples in a short time span and is vulnerable enough to distinguish active ingredients at low concentrations [23]. Figure 1 highlights the DPPH radical scavenging ability of *E. suberosa* bark extract at different concentrations. Although the DPPH radical scavenging activities of the extract was found less than those of the ascorbic acid, the study had made a disclosure that *E. suberosa* bark extract has concentration- dependant free radical scavenging or inhibition activity, possibly acting as primary antioxidants. There was an observation on a similar trend in a study of the antioxidant activity of the *Artemisia judaica* L. extract [24].

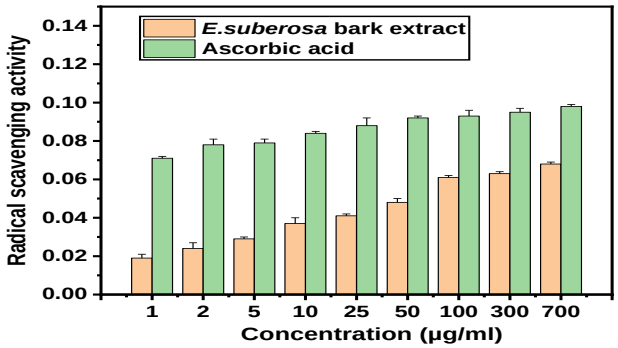

Figure. 1. Radical scavenging or antioxidant activity of crude chloroform extract of *Erythrina suberosa*. Results are expressed as a mean ± SD.

3.3. Anticancer Activity

A series of MTT assay was conducted to explore the cytotoxic effect of *E. suberosa* bark extract against Ht144 cell line. Results from MTT assays show that *E. suberosa* bark extract exhibited concentration dependent cytotoxic effect against the investigated cell lines. As depicted in figure, the results revealed that the *E. suberosa* bark extract significantly inhibited the growth of the HT-144 cells with IC_{50} value 77.48 mg/ml in comparison with untreated group ($P < 0.05$). In addition, the *E. suberosa* bark extract show its best performance at high concentration of about 400 mg/ml, as depicted in figure 2.

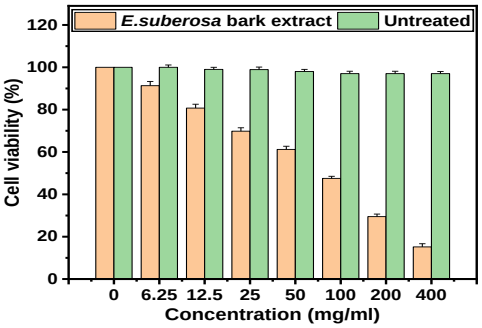

Figure. 2. Cytotoxicity assay of crude chloroform extract of *Erythrina suberosa*. Untreated group was used as control. Results are expressed as a mean ± SD.

3.3.4. *In-vivo* anti-inflammatory activity of *E. suberosa* bark extract

In-vivo anti-inflammatory activity of *E. suberosa* bark extract was evaluated in rats. As shown in figure 3, rats in experimental group administrated with 100 mg/kg dose has shown less symptoms of edema good as compared to diseases and control group, suggest anti-inflammatory effect of *E. suberosa* bark extract . Further we observed that rats treated with 200 mg/kg and 400 mg/kg dose has shown better inflammatory effect than 100 mg/kg dose treated rats, indicated that *E. suberosa* bark extract exhibited concentration-dependant anti-inflammatory in rats. DMSO, 1% carrageenan, and diclofenac sodium treated rats were used as control, diseased and standard group, respectively.

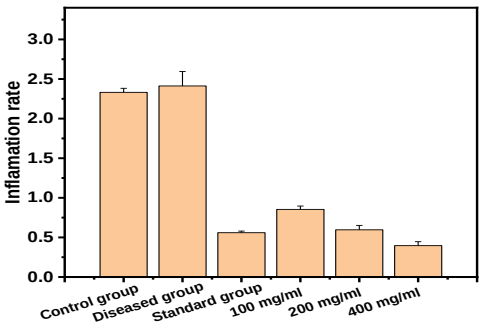

Figure. 3. Anti-inflammatory effect of *E. suberosa* at concentrations of 100 mg/kg , 200 mg/kg, 400mg/kg and its comparison with control group (DMSO), disease group and standard group (diclofenac sodium). Data are expressed as a mean ± SD.

FTIR studies of *Erythrina suberosa* bark extract

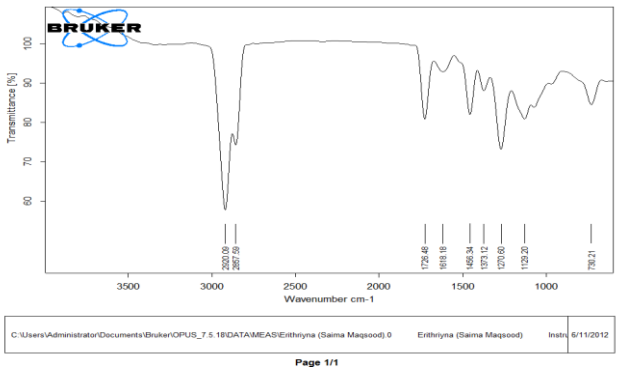

Figure 4: FTIR spectra of pure *E. suberosa* bark extract.

Table 2. Spectral peak values and functional groups obtained from the chloroform bark extract of *E. suberosa*.

Peak Values	**Functional Groups**
2920.09	C-H stretching
2857.39	C-H aldehydic
1726.48	C=O aldehyde
1618.18	C=C alkene
1456.34	C=C aromatic
1373.12	NO_2 stretch
1270.60	C-O
1129.20	C-F
730.21	C-Br

GCMS studies of *Erythrina suberosa* bark extract

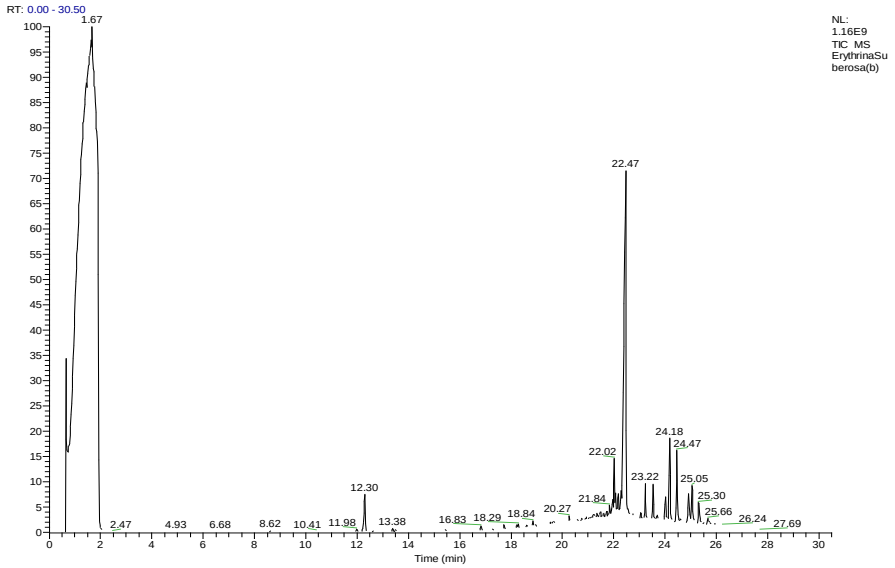

Figure 5. GC-MS Chromatogram of chloroform extract of *E. suberosa* bark extract.

Table 3. Compound name, molecular formula and molecular weight of compounds of E. suberosa bark extract.

No	Compound Name	Molecular Formula	Molecular weight
1	Trichloromethane	CHC_{l3}	118
2	Trichloromethane	CHC_{l3}	118
3	Imipramine	$C_{19}H_{24}N_2$	280
4	Clomipramine	$C_{16}H_{32}O_2$	256
5	5,7-dimethylundecane	$C_{13}H_{28}$	184
6	D-Glycero-d-ido-heptose	$C_7H_{14}O_7$	210
7	Benzene, (1-methylnonadecyl)-	$C_{26}H_{46}$	358
8	Tert-Hexadecanethiol	$C_{16}H_{34}S$	258
9	Octadecane, 1-chloro-	$C_{18}H_{37}Cl$	288
10	Nonadecane	$C_{19}H_{40}$	268
11	Pentadecane	$C_{15}H_{32}$	212
12	Isoproturon	$C_{12}H_{18}N_2O$	206
13	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	240
14	Aspidospermidin-17-ol	$C_{23}H_{30}N_2O_5$	414
15	Octadecane, 1-chloro	$C_{18}H_{37}Cl$	288
16	2-Hexenoic acid, 5-hydroxy-3,4,4-trimethyl-, (E)-	$C_9H_{16}O_3$	172
17	Octadecane, 1-chloro-	$C_{18}H_{37}Cl$	288

18	Pentadecane, 2,6,10-trimethyl-	$C_{18}H_{38}$	254
19	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206
20	1-Hexadecene	$C_{16}H_{32}$	224
21	Octadecane, 1-chloro-	$C_{18}H_{37}C$	288
22	2-Naphthalenemethanol, decahydro-à,à,4a-trimethyl-8-methylene-	$C_{15}H_{26}O$	212
23	1-Chloroeicosane	$C_{20}H_{41}Cl$	316
24	Pentadecane, 8-heptyl	$C_{22}H_{46}$	310
25	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366
26	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	256
27	18,19-Secoyohimban-19-oic acid, 16,17,20,21-tetradehydro-16-(hydroxy methyl)-, methyl ester, (15á,16E)-	$C_{21}H_{24}N_2O_3$	352
28	Phthalic acid, butyl tetradecyl ester	$C_{26}H_{42}O_4$	418
29	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl] cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester	$C_{25}H_{42}O_2$	374
30	1-Docosene	$C_{22}H_{44}$	308
31	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	294
32	18,19-Secoyohimban-19-oic acid, 16,17,20,21-tetradehydro-16-(hydroxy methyl)-, methyl ester, (15á,16E)-	$C_{21}H_{24}N_2O_3$	352

33	18,19-Secoyohimban-19-oic acid, 16,17,20,21-tetradehydro-16-(hydroxy methyl)-, methyl ester, (15á,16E)-	$C_{21}H_{24}N_2O_3$	352
34	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	$C_{26}H_{42}O_4$	418
35	Morphinan, 7,8-didehydro-4,5-epoxy-3,6-dimethoxy -17-methyl-, (5à,6à)-	$C_{19}H_{23}NO_3$	313
36	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390
37	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382
38	Ethyl tetracosanoate	$C_{26}H_{52}O_2$	396
39	Hexacosanoic acid, methyl ester	$C_{27}H_{54}O_2$	410
40	Hexacosanoic acid, 2-methyl-, methyl ester	$C_{28}H_{56}O_2$	424
41	Octacosanoic acid, methyl ester	$C_{29}H_{58}O_2$	438
42	Cholesta-22,24-dien-5-ol, 4,4-dimethyl-	$C_{29}H_{48}O$	412
43	17-Pentatriacontene	$C_{35}H_{70}$	490
44	17-Pentatriacontene	$C_{35}H_{70}$	490

4. Discussion

The major objective of using plant as a source is to separate active components of plants. These active components were used as lead compound for synthesis of further products which are pharmacologically highly active and have low toxicity [25]. Literature review enlightens that expectorant, bronchodilator, laxative, spasmolytic, anthelmintic, diuretic, and emmenagogue properties of *E.suberosa* were determined so the present study was carried to found the antioxidant, anticancer and anti-inflammatory activities [26]. Scavenging ability of antioxidants was measured through DPPH assay. As antioxidants donate protons to the radical, the absorption decreases. Antioxidants on interaction with DPPH transfer either an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. As antioxidants donate protons to radical, the absorbance decreases [27].The absorbance of different concentration of *E.suberosa* and vitamin C was measured by using UV spectrophotometer and their scavenging activity results were compared. The antioxidant activity of test compound at 700 microgram concentration is high as compared to other doses but it is far less than standard compound.

The unrepairable damage of cells resulted in cancer and further division of these damaged cells is cause of tumor. Skin cancer varies with different type of skin and its development also depend on heredity [28].Cancer is a group of more than 100 different diseases characterized by uncontrolled cellular growth, local tissue invasion. Bark extract of *E. variegata* has been used to investigate anticancer activity [29]. In this study anticancer activity of *E.suberosa* was found. Figure 3 shows the anticancer activity of *E. suberosa* against human fibroblast malignant melanoma through MTT assay. Plant extract shown the strong growth inhibition (15.187261.1 ± 1.13217) against the cell lines after the exposure of 24 hours. Inflammation is a defensive mechanism of our body against infectious particles but it is essential to remove symptoms related with inflammation. For this purpose anti-inflammatory agents were used which can be synthetic or natural drugs. Synthetic agents has severe side effects including stomach ulcer, and bleeding etc so we had tried to replace synthetic agent with other products which were obtained from medicinal plants having good efficacy and low toxicity [30]. The anti-inflammatory property was evaluated by using rats. Different concentration of oral doses (100 mg/kg, 200 mg/kg and 400 mg/kg) were given to rats and their results were compared with control group, diseased group and standard group. It was observed that rats administrated with 400mg/kg dose has shown the maximum anti-inflammatory activity as compared to other doses.

5. Conclusion

The current research studies has concluded that *E.suberosa* bark extract is the potential source of cytotoxic compounds as it has shown good results against cancer cell lines HT-144. *E. suberosa* 400mg/kg dose has good anti-inflammatory activity and moderate antioxidant activity. *E.suberosa* require further studies to proclaim pharmacologically active lead compounds.

Declarations of competing interest

The authors declared no competing interest.

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