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Phytochemical and GCMS approaches to identify active constituents in *Erythrina suberosa* bark extract and evaluation of its therapeutic potency

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Abstract: Humans rely on plants as a necessitous source of their food, energy, cosmetics and medicines, as medicinal plants are rich source of new therapeutically active compounds from decades. Current study was designed to separate and identify active constituents of *Erythrina suberosa* bark extract using phytochemical screening and gas chromatography and mass spectroscopy, respectively and evaluated their therapeutic activities. *E. suberosa* bark extract contained saponins, glycosides, alkaloids, tannins, terpenoids, phenols and 44 active compounds identified by phytochemical and gas chromatography and mass spectroscopic analysis. Therapeutic potentials of *E. suberosa* bark extract was evaluated by such as cytotoxicity, anti-inflammatory and antioxidant assay. Surprisingly, bark extract shows the concentration dependent cytotoxicity against human fibroblast malignant melanoma-144 cell lines and remarkably inhibited (15.18±1.13%, at 400mg/ml) growth of cancer cells after 24 hours treatment. In addition, the *E. suberosa* bark extract also exhibited anti-inflammatory effect at higher doses (400mg/kg) and moderate antioxidant activity is also noticed through (2, 2-diphenyl-1-picrylhydrazyl radical) assay. These findings indicate that *E. suberosa* bark extract exhibited prominent anticancer and anti-inflammatory activities and might be serve as a potent therapeutic agent in future.

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

INTRODUCTION

Medicinal plants and shrubs have been applied in healthcare since ancient time. Herbal medicines are considered to be more affordable, easily available, safer and free of toxic side effects disparate to their synthetic counterparts. In developing countries about 3.3 billion peoples utilize medicinal plants for the treatment of different diseases (Cordell, 2011, Sofowora, 2013). According to WHO, traditional herbal medicines and phytonutrients or nutraceuticals are getting significant attention in health debates worldwide (Organization, 2004). About 80% of African population uses herbal medicine for healthcare and the per annum global market value of phytoproducts exceeds $100 billion.

In Canada, France and India 50%, 75% and 65% of the population respectively, depends on herbal medicine alone or in combination with conventional pharmaceuticals. Furthermore, 85% of Japanese doctors prescribe traditional herbal medicine, instead of modern medicine (Aschwanden, 2001). In China, about 40% of all healthcare services provided in China depends on herbal medicine. Moreover, herbal medicine played a significant role in the treatment strategy of respiratory diseases such as severe acute respiratory syndrome (SARS) and COVID-19 (Afzal, 2021). *Erythrina suberosa* is a medium size (10 m) deciduous plant commonly known as corky coral or Indian coral tree, belongs to pea family and widely used for ornamental purpose in Pakistan. *E. suberosa* is a rich source of secondary metabolites, particularly flavonoids, phenolics and alkaloids that exhibit promising anti-inflammatory, antiplasmodial, bactericidal and fungicidal activities, respectively. Alkaloids, extracted from *E. suberosa* flowers displayed a remarkable anxiolytic effect *in-vivo* (Flausino *et al*., 2007).

In another study, Guaratini *et al* reported the strong anxiolytic activity of erythraline alkaloid by blocking or inhibiting α-4/β-2 nicotinic receptors (Guaratini *et al*., 2014). Furthermore, the crude aqueous extract of *E. suberosa* flowers showed potential bronchodilators, spasmolytic and antioxidant properties (Janbaz *et al*., 2020). The methanolic aqueous extract of *E. suberosa* of stem bark contain certain alkaloids and saponins which exhibited prominent antibacterial, antifungal and cytotoxic effects against human pathogens (Ahmed *et al*., 2020). Current study focused on to identify the active constituents of *E. suberosa* bark extract by phytochemical screening and GCMS and evaluated it's an anti-inflammatory, anticancer and antioxidant activities *in vitro* and *in vivo*.

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MATERIALS AND METHODS

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 of Department of Pharmaceutical Chemistry, Riphah International University, Islamabad, Pakistan, where a voucher sample was placed for reference purpose.

Crude extract preparation

The collected plant materials were carefully rinsed with water to remove the contaminants followed by shade drying to minimize the chances of losing light sensitive active components. The dried bark was cut into small and soaked in a percolator containing chloroform for two weeks. Subsequently, the mixture was filtered through grade no. 1 Whatman filter paper to collect the extract. Next, chloroform was evaporated by rotary evaporator at 40°C under reduced pressure. The obtained concentrated extract was stored of 4°C for further 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 (Madureira *et al*., 2012).

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, terpenoids and phenols, by using different reagents as mentioned (Khan and Rashid, 2006, Serrano *et al*., 2011, Juma and Majinda, 2004, Mohanta *et al*., 2017).

Test for saponins

Briefly, *E. suberosa* bark extract was placed in 2.0ml tube and subsequently a small quantity lead acetate was gently added and observe extract solution for any colour change.

Test for cardiac glycosides

Crude extract of *E. suberosa* bark was taken in a test tube and treated with glacial acetic acid (2ml). Further add sulphuric acid (1.0ml) and $FeCl_3$ (1.0ml) to the test tubes. Observe the formation of reddish black layer for the presence of cardiac glycosides.

Test for alkaloids

E. suberosa bark extract solution (1.0ml) was acidified with aqueous acetic acid solution (10%) in two separate test tubes A & B followed by the addition of Mayer's reagent and Dragendorff's reagent, respectively. The appearance of cream-colored precipitates with Mayer's reagent and reddish-brown precipitate with Dragendorff's reagent confirm the presence of alkaloids.

Test for tannins

Few drops of $FeCl_3$, 5% solution were gently added to crude extract of *E. suberosa* bark, and observe for colour any colour change.

Test for terpenoids

Crude extract of *E. suberosa* bark was taken in test tubes (5.0ml) and supplied with chloroform (3.0ml) followed by the addition of concentrated H_2SO_4 (3.0ml). The presence of terpenoids was confirmed by the reddish-brown colour of the interface.

Test for flavonoids

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

Test for phenols

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

Characterization of E. suberosa Bark Extract

Fourier transform infrared (FTIR) spectrum of extract was measured in range of range of wavenumber 500-4000 cm^{-1} by directly placing the sample on the crystal plate centre of FTIR spectroscope (Perkin Elmer, USA). The resultant peaks were compared with the previous reported studies (Caunii *et al*., 2012, Cao *et al*., 2017, Mohamed *et al*., 2017). GCMS technique has been used for identification of active compounds present in crude extract of *E. suberosa* bark.

Biological Evaluation of E. suberosa Bark Extract

Antioxidant activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl radical) assay was used for determination of antioxidant activity of test sample. Briefly, various concentrations (2, 5, 10, 25, 50, 100, 300, and 700µg/ml) of test sample chloroform were added to equal volume methanolic DPPH radical, mixed for 1.5min and *UV* spectrum was recorded by spectrophotometer (UV-2600i Shimadzo) at 517 nm wavelength. Ascorbic acid (vitamin C) was used as standard antioxidant agent. The radical scavenging activity of test sample was calculated using formula:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of standard} - \text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

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 using previously reported method with some modifications (Iqbal *et al*., 2020). Briefly, HT-144 (5×10^5 cells/well) were cultured in 96-well plate supplied 100µL of growth medium in to each well and incubated for 24h. After incubation, cell were supplied with fresh medium containing serial concentrations (ranges from 0 - 400mg/ml) of test sample and further incubated for 24. After treatment, cells were

washed with PBS and supplied with fresh medium (120 µl) containing 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 to formazan crystals. 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.

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

Anti-inflammatory activity

Anti-inflammatory potential of *E. suberosa* was determined through carrageenan induced rat hind paw edema model. The rats were provided by the animal house of pharmacology department of Riphah Institute of health sciences. Rates were divided into four groups, namely control, diseased, standard, and experimental groups and each group contain equal rats. Control group (Group I) was treated with 0.4% DMSO, diseased group (Group II) was treated with 1% carrageen an, standard group (Group III) was intraperitoneally treated with 10mg/kg diclofenac before carrageenan treatment. Experimental group (Group IV) was further divided into three groups. Rats of group (a), (b), (c) were given with 100mg/kg, 200mg/kg and 400 mg/kg oral dose of test compound respectively for 5 days. On 5th day of experiment carrageenan was given intradermally in hind paw after 30 mins of test sample 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.

STATISTICAL ANALYSIS

The experiments were reproduced thrice independently to ensure the consistency of the data. T-tests was applied. The values were expressed as the mean ± standard deviation (SD). One way analysis of variance (ANOVA) was applied to analyse the data followed by post hoc Bonferroni multiple comparison using Graph Pad Prism-6 (San Diego, CA, USA). Symbol * shows the significant difference relative to disease group and # represents the significant difference relative to saline group. $p<0.05$ was recommended as statistically significant.

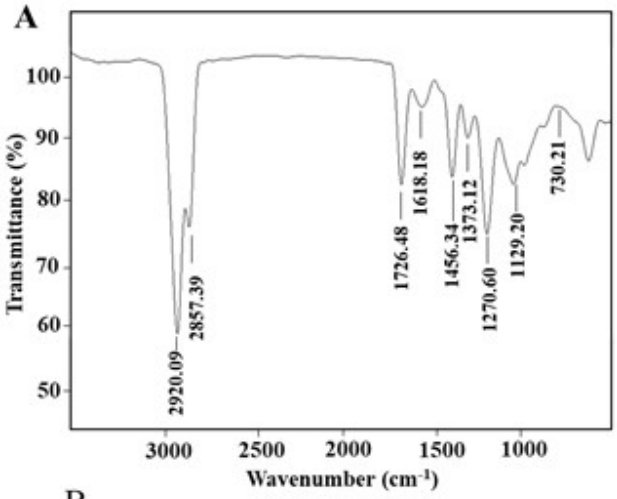
RESULTS

Phytochemical evaluation

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.

Characterization of E. suberosa Bark Extract

The FTIR spectrum is widely used to identify the functional groups of active constituents present in plant extracts based on their respective peak values in IR region. FTIR spectrum results validated the presence of several active chemical constituents on the basis functional groups such as alkenes, aromatic carboxylic acid, alcohol, alkyl halide, and halogen compound in *E. suberosa* bark extract shown fig. 1 A & B.



B

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

Fig. 1: A) FTIR spectrum of *E. suberosa* Bark Extract. B) Corresponding peaks with functional groups

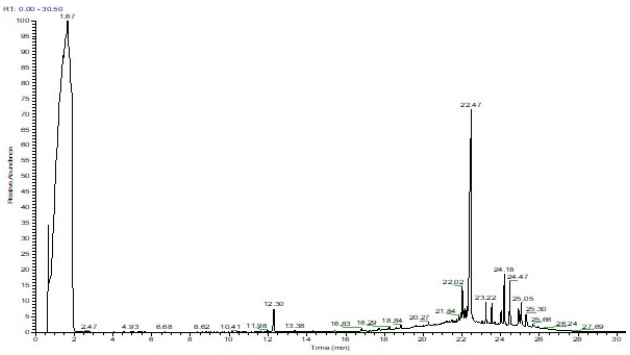


Fig. 2: GCMS spectrum of *E. suberosa* bark extract.

GCMS analysis of E. suberosa bark extract

GCMS technique is specially used to identify the active constituents in plant extarct such as volatile matter, esters long chain, branched chain hydrocarbons, alcohols acids,

etc. The GCMS spectrum of *E. suberosa* bark extract showed the presence of twenty four (24) phytochemical compounds (active constituents) that might contribute to the therapeutic function of the *E. suberosa* bark extract shown in fig. 2. The phytochemical compounds in *E. suberosa* bark extract was confirmed on the bases of peak area, molecular weight and molecular formula. The active constituents with compound name, molecular formula, and molecular weight are shown in table 2.

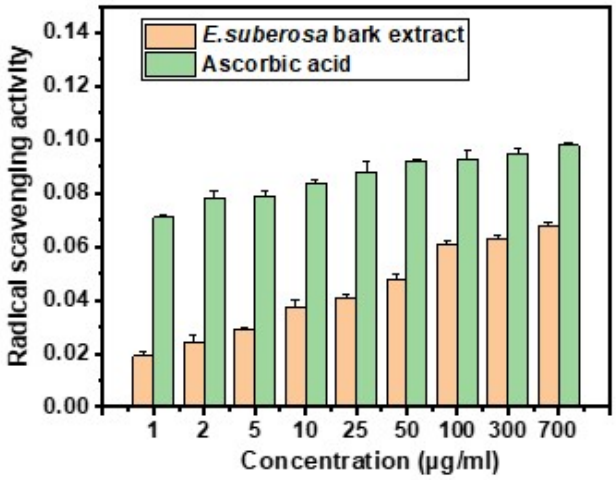


Fig. 3: Radical scavenging or antioxidant activity of crude chloroform extract of *Erythrina suberosa*. Results are expressed as a mean ± SD.

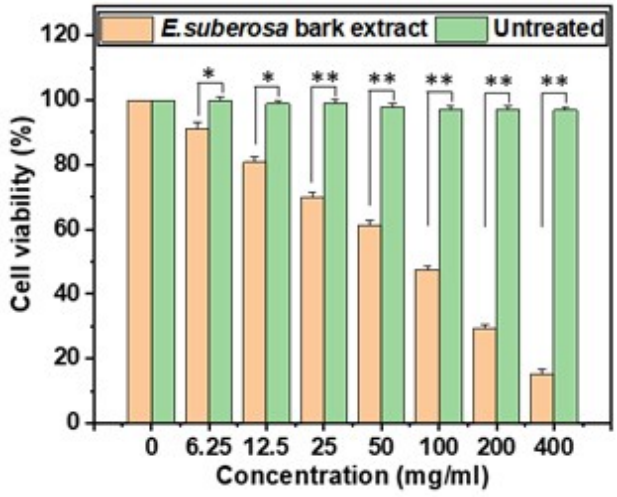


Fig. 4: Cytotoxicity assay of crude chloroform extract of *Erythrina suberosa*. Untreated group was used as control. Results are expressed as a mean ± SD. *p<0.05, **p<0.01 indicated significant difference compared to control group (untreated).

Antioxidant activity

DPPH radical has been widely used as a probe for rapid assessment of antioxidant activity of plant extracts. The abrupt decrease in DPPH absorbance occurred after accepting hydrogen radical or electron which might causes change in colour from purple to yellow (Hseu *et al*., 2008). As shown in fig. 3, radical scavenging activity of DPPH radical increased as *E. suberosa* bark extract concentration increased, which may due to phenolic compounds in *E. suberosa* bark extract. The DPPH radical scavenging activity of the ascorbic acid (Vitamin C) was found high than our tested sample, however we explored that *E. suberosa* bark extract exhibited radical scavenging activity, which can be possibly used as primary antioxidants. Our results are highly consistent with previous reports (Liu *et al*., 2009).

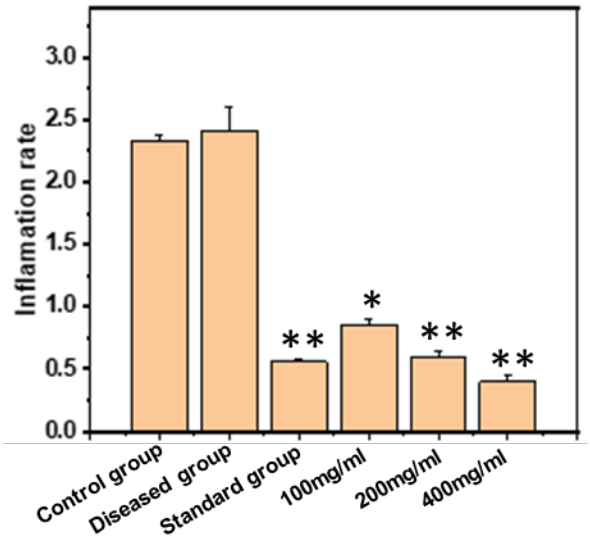


Fig. 5: Anti-inflammatory effect of *E. suberosa* at concentrations of 100mg/kg, 200mg/kg, 400mg/kg and its comparison with control group (DMSO), disease group and standard group (diclofenac sodium). Data are expressed as a mean ± SD. The *p values < 0.05, and **p values < 0.01 were considered statistically significant compared control and diseased group.

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 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assays show that *E. suberosa* bark extract exhibited concentration dependent cytotoxic effect against the investigated cell lines shown in fig. 4. The results revealed that the *E. suberosa* bark extract significantly inhibited the growth of the HT-144 cells with IC_{50} value 77.48mg/ml in comparison with untreated group (p<0.01). As shown in fig. 4, the anticancer activity of *E. suberosa* bark extract significantly increased with increase in concentration, and maximum at 400 mg/ml.

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 fig. 5, Further

Table 1: Phytochemical analysis of crude extract of *Erythrina suberosa* bark

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 2: Compound name, molecular formula, and molecular weight of identified active compounds in *E. suberosa* bark extract.

No.	Name of the compound	Molecular Formula	Molecular Weight
1	2-Hexenoic acid, 5-hydroxy-3,4,4-trimethyl-, (E)-	$C_9H_{16}O_3$	172
2	5,7-dimethylundecane	$C_{13}H_{28}$	184
3	Isoproturon	$C_{12}H_{18}N_2O$	206
4	D-Glycero-d-ido-heptose	$C_7H_{14}O_7$	210
5	Pentadecane	$C_{15}H_{32}$	212
6	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	240
7	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
8	Tert-Hexadecanethiol	$C_{16}H_{34}S$	258
9	Nonadecane	$C_{19}H_{40}$	268
10	Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280
11	9-Octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	282
12	Octadecane, 1-chloro-	$C_{18}H_{37}Cl$	288
13	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	294
14	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_3O_2$	296
15	1-Docosene	$C_{22}H_{44}$	308
16	Morphinan, 7,8-didehydro-4,5-epoxy-3,6-dimethoxy -17-methyl-, (5à,6à)-	$C_{19}H_{23}NO_3$	313
17	1-Chloroeicosane	$C_{20}H_{41}Cl$	316
18	Diethylhexadecanedioate	$C_{20}H_{38}O_4$	342
19	Benzene, (1-methylnonadecyl)	$C_{26}H_{46}$	358
20	Di-n-octylphthalate	$C_{24}H_{38}O_4$	390
21	Aspidospermidin-17-ol	$C_{23}H_{30}N_2O_5$	414
22	Friedelan-3-ol	$C_{30}H_{50}O$	426
23	Octacosanoic acid, methyl ester	$C_{29}H_{58}O_2$	438
24	17-Pentatriacontene	$C_{35}H_{70}$	490

we observed that rats injected with 200mg/kg and 400mg/kg dose has shown better inflammatory effect than 100mg/kg dose treated rats, indicated that *E. suberosa* bark extract exhibited concentration-dependant anti-inflammatory in rats. Dimethyl Sulfoxide (DMSO), 1% carrageenan, and diclofenac sodium treated rats were used as control, diseased and standard group, respectively.

DISSCUSSION

Discovery of new drugs from medicinal plants and shrubs have played a vital role in the management of different infections. Indeed, a secondary metabolite of medicinal plants and their derivatives have been used to combat cancer and microbial infection, over the last century. Plant extracts provides treatment to all cancer phases as they are multi targeting and induce synergistic effect (Fabricant and Fansworth, 2001). Previously, different activities of *E. suberosa* extract i.e., expectorant, bronchodilator, laxative, spasmolytic, anthelmintic and diuretics (Patti *et al*., 2019). In the present study, first we analysed *E. suberosa* bark crude extract using different phytochemical test to identify active constituents. We found that crude extract comprised of alkaloids, flavonoids, saponins, glycosides and phenolic compounds. Later on, FTIR analysis also confirmed the attributed function group of alkaloids, flavonoids, saponins, glycosides and phenolic compounds, which

might be responsible for biological activities of crude extract. GCMS analysis identified 24 active chemical compounds (table 2) in *E. suberosa* bark, and the identified compounds exhibited various biological activities. The moderate antioxidant activity of *E. suberosa* bark extract might be due the presences of n-hexadecanoic acid known for moderate antioxidant and strong anti-inflammatory activity previously identified in pteleopsis suberosa extract (Sanni and Omotoyinbo, 2016).

Hexadenoic acid has also been reported as active component in alcoholic leaves extract of *Melissa officinalis* and *Kigelia pinnata* (Sharafzadeh *et al*., 2011, Grace *et al*., 2002). Moreover, the *E. suberosa* bark extract possess strong anticancer activity at concentration of 200 and mg/ml, this strong anticancer effect is may be due the presence of flavonoids containing active compounds such as alpinumisoflavone and licoflavanone previously identified by Kumar *et al*., using *E. suberosa* stem bark extract (Kumar *et al*., 2013). Our reported results is highly consistent with previously reported results for plant derived secondary metabolites. In this we also reported the strong anti-inflammatory activity at 400 mg/kg orally administered dose which might be due the other GCMS identified compounds such as 9-Octadecenoic acid (Z)- and 9, 12-Octadecadienoic acid (Z,Z) - both fatty acid ester and methyl ester possess strong anti-inflammatory effect. Other compounds as identified by GC-MS in chloroform extract possess various pharmaceutical applications. In short, *E. suberosa* bark extract show strong anticancer, anti-inflammatory effect and moderate antioxidant effect due its identified active compounds which might be used as a potent therapeutic agent in future.

CONCLUSION

Specific quantitative and qualitative patterns from phytochemical and GCMS analyses usually help to identify bioactive components and their plant source. The current study reveals the presence of bioactive constituents *in E. suberosa* and further explored that the identified Phyto-components exhibited strong anticancer and anti-inflammatory effect. However, moderate anti-oxidant activity has also been observed. Hence, further studies still need to be conducted on these identified bioactive components to evaluate their individual medicinal activities, which might be necessary for the development of safe drugs of interest.

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