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A potent chemopreventive effect of diethyl ether extract of *lotus peregrinus* L. on apoptosis associated proteins and oxidative stress markers in mammary tumor –induced in rats

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A B S T R A C T

The objective of the present study was to investigate the bioactive constituent of diethyl ether extract and to determine whether the diethyl ether extract of Lotus peregrinus L. exerts chemo-protected effect by stimulating apoptosis during medroxyprogesterone acetate (MPA)-DMBA mammary carcinogenesis using the expression of the apoptosis-associated proteins Bax and caspases 9; as well as improvement of oxidative status of the mammary tumor tissue. Sixty female wistar rats (52 days old) were divided into four equal groups. Group I (normal group): rats administered distilled water. Group II (carcinogenic group); on day 0, wistar rats were given DMBA (2.5 mg/ rat; orally) followed by second dose DMBA (2.5 mg/ rat; orally) on day 15, 90-day timed-release pellets containing 25 mg MPA were implanted' into rats on day 30. Group III (diethyl ether protected), on day 0, wistar rats were given DMBA (2.5 mg/ rat; orally) followed by second dose DMBA (2.5 mg/ rat; orally) on day 15, on day 26 rats were administered diethyl ether (50 mg /kg. b. wt.; orally), 90-day timed-release pellets containing 25 mg MPA were implanted into rats on day 30. Group IV (diethyl ether treated group); given diethyl ether (50 mg /kg. b. wt.; orally) after induction of mammary cancer. Our findings showed the positive treatment of mammary tumor and improved oxidative status of the tissue was evident by increase in the levels of GSH, Catalase and decreased levels of serum MDA levels which in turn is an indicator of reduced oxidative stress of the tissue. Therefore, this study suggests that diethyl ether extract of *Lotus peregrinus* can be useful for the treatment as well as improvement of oxidative status of the mammary tumor tissue .Also, was more effective in inhibiting DMBA-MPA induced mammary tumors and modulating the expression of apoptosis associated proteins.

Key words: *Lotus peregrinus L., Diethyl ether extract, Caspase 9, Bax and Oxidative status*

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1. INTRODUCTION

Breast cancer is an uncontrolled growth of breast cells originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules as a result of mutations in the genes responsible for regulating the growth of cells and keeping them healthy. Worldwide 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths occurred in 2008.The estimated number of new breast cancer cases has been raised from about 641,000 cases in 1980 to 1.6 million cases in 2010 and 625,000 deaths in 2010 (Jemal *et al*., 2011) . Breast cancer is the most common cancer of adult females all over the world (Aguas *et al.*, 2005), and after lung cancer, it is the second leading cause of cancer death (Dumitrescu and Cotarla, 2005). Breast cancer is known to have a long latency period; there may be several decades between the initiation of the carcinogenic process and clinical detection (Ostrowski *et al*., 1999). The medicinal values of plants lie in their bioactive chemical contents that produce a definite therapeutic benefit on the human body (Edeoga *et al*., 2005). These are non-nutritive substances, have protective or disease preventive property (Ahmed and Urooj, 2009). The most important of these bioactive compounds are alkaloids, flavonoids, tannins and phenolic compounds (Purkayastha and Dahiya, 2012). These are the important raw materials for drug production (Tullanithi *et al*., 2010). Fabaceae is the most common family found in tropical rainforests and in dry forests in America and Africa (Burnham and Johnson, 2004). Many species of this family had medicinal values and used in folk medicine. Isoflavone lupiwighteone isolated from Lotus pedunculated induces anticancer, apoptotic, and antiangiogenic activities (Ren *et al*., 2015). In addition, (Ibrahim, 2016) showed that *Lotus halophilus* successive extracts with different concentrations were tested against MCF7 (Breast carcinoma cell line) showing high cytotoxic value. Breast cancer is associated with altered antioxidant enzyme activities and cellular redox status, excessive cell proliferation, dysregulation of cellular differentiation, and insufficient apoptosis (Kumaraguruparan *et al.*, 2007). Elevated levels of reactive oxygen species, DNA damage, lipid peroxidation, and reduction of total antioxidant capacity have also been associated with breast cancer (Tas *et al*., 2005). Using a transgenic mouse model system (Shibata *et al.*, 1999) demonstrated a role for Bax in inhibiting mammary cancer progression. An analysis of Bax expression in human breast cancer specimens revealed the localization of Bax in normal mammary epithelium. Bax-alpha, a splice variant of Bax that promotes apoptosis, is expressed in high amounts in normal cell lines and breast tissue but is expressed only weakly or not at all in cancer cell lines and malignant tissue (Bargou *et al*. 1995). B-sitosterol supplementation to MDA-MB-231 cells induces 39% and 80% increases in the activities of caspases 8 and 9. There was also a 3-fold increase in the activity of caspase 3. It is concluded that β-sitosterol may induce apoptosis through the intrinsic pathway (Awad *et al.*, 2003).

Apoptosis is characterized by distinct morphological and biochemical features, such as nuclear and cytoplasmic condensation, DNA fragmentation, dilation of the endoplasmic reticulum, alterations in the cell membrane composition, and formation of membrane-enclosed apoptotic bodies (Kerr *et al*, 1972). Initiation of apoptosis involves the activation of cysteine-dependent aspartyl proteases known as caspases, Caspases are constitutively present in cells in an inactive precursor form that must then be cleaved and processed for activation ; Initiator caspases (caspases-8, and -9) activate effector caspases (caspases-3, -6 and -7), which then cleave structural and regulatory proteins such as DNA fragmentation factor-45 (DFF45), poly (ADP-ribose) polymerase (PARP), lamins, and cyto-keratins, and ultimately result in the organized destruction of the cell (Earnshaw *et al.*, 1999; Slee *et al.*, 1999; Bratton *et al.*, 2000; Sun *et al*., 1999).

2. MATERIALS AND METHODS

2.1. Plant Materials

The fresh Lotus peregrinus L. aerial parts were collected from Wadi Habis (Mersa Matrouh governorate), Egypt, during spring season; 2015. The collected plants were identified by Botany Department, Faculty of Science, Cairo University and by comparison with plant description in flora of Egypt as well as herbarium specimens at Desert Research Center (Egypt). The aerial parts were air-dried under shade then dried in an oven at 40°C till constant weight. Finally, grinded to fine powder.

2.2 Preparing of diethyl ether extract of Lotus peregrinus:

About 2 kg of air-dried aerial parts of plant materials were subjected to successive extraction using different organic solvents using separating funnel. Diethyl ether, chloroform, ethyl acetate, 70 % ethanol and water, solvents were used in the order of increasing polarity for the same quantity of the plant powder. The obtained residue from each solvent was dried and weighed (*Ibrahim, 2016*).

2.2 Determination of median lethal dose (LD_{50}) of diethyl ether extract of Lotus peregrinus:

Five doses (start dose at 5 mg/kg) followed by three 10-fold doses and finally 2-fold dose at 2000 mg/kg body weight) were chosen for the determination of oral LD50 in rats (Table 1) and given to five groups of albino rats (10 in each group). The animals were observed for first 2 hours and then at 6th and 24th hour for any toxic symptoms. The number of rats that survived were noted after 24 h and then maintained for the further 13 days with daily observation for any further toxicity. The toxic effects of diethyl ether extract of *Lotus peregrinus* L. was assessed on the basis of mortality, which was expressed as LD_{50}. Median lethal dose (LD_{50}) was calculated using a dose response curve (plotting percent mortality on Y axis and dose on X axis) method by GraphPad prism software.

2.3 Terpenoid contents of diethyl ether extract of Lotus peregrinus L.

Gas chromatography - mass spectrometry (GC-MS) analysis was performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica HP-1 capillary column (30 m × 0.25 mm i.d.; 0.33 μm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization energy voltage 70 eV; electron multiplier voltage energy 2000 V. Mass spectra were scanned in the range 35–450 amu, scan time 5 scans/s. Gas chromatographic conditions were as reported above; transfer line temperature, 295°C (*Eltantawy, 2017*).

2.4 Hydrocarbon and sterols contents of diethyl ether extract of Lotus peregrinus L:

The investigation of Hydrocarbon and sterols were carried out qualitatively and quantitatively by using Gas Liquid Chromatography. The results of Itoh *et al.,* (1973) and Farag *et al.,* (1986) were used as a guide to characterize some of the unknown compounds. The relative percentage of each Hydrocarbon and sterol compound was determined using triangulation method according to Nelson *et al.,* (1969).

2.5 Extraction and Identification of Phenolic Acids of diethyl ether extract by HPLC:

Phenolic compounds of *Lotus peregrinus* plant sample were extracted according to the method outlined by Ben-Hammouda *et al.* (1995). Phenolic compounds of the sample were identified by comparing their relative retention times with those of the standards mixture chromatogram. The concentration of an individual compound was calculated on the basis of peak area measurements. All

chemicals and solvents used were HPLC spectral grade.

2.6. Experimental animals:

Sixty white female wistar rats of 52 day old and weighing 60-80 g were used in this study. Rats were housed in separated metal cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The rats were fed on constant ration and fresh, clean drinking water was supplied ad-libitum. All rats were acclimatized for minimum period of two weeks prior to the beginning of study.

2.7. Chemicals used:

All chemicals were of analytical grade and obtained from standard commercial suppliers. Chemicals used in the present study were

a. DMBA: 7, 12 dimethyl-benz[a]anthracene purchased from Sigma Aldrich Company. Administered orally at a dose of 2.5 mg / rat; (double dose); first at day 0 and second at day 15 (Macejova and Brtko, 2001).

b. Diethyl ether: purchased from Elgomhoria Company, Freshly prepared with dry powder of *Lotus peregrinus* plant administered orally three times per weeks at a dose of 50 mg/kg b.wt (1/20 of LD_{50}).

c. MPA: Medroxy progesterone acetate .it administered intra-muscular to rats at a dose level (25 mg /rat / i.m) once at day 30 *(Candace et al., 2010).*

d. The experiment demonstrated-that antioxidants can inhibit the development of mammary tumors of rats initiated with DMBA.

2.8. Experimental design:

After acclimatization to the laboratory conditions, the animals were randomly divided into three groups (14 rats each) placed in individual cages and classified as follow:

a. Group I (normal control group): Rats received no drugs, served as control non-treated for all experimental groups.

b. Group II (chemically induced mammary cancer group): Rats received DMBA at a dose level of 2.5 mg/rat at day 0 and second dose 2.5 mg/rat at day 15 followed by MPA at a dose level 25 mg / rat at day 30.
c. Group III (diethyl ether extract of *Lotus peregrinus* protected group): Rats received diethyl ether extract (50 mg/kg b.wt) orally three times per weeks at day 26 from beginning of experiment till the end of treatment (22 weeks).
d. Group IV (diethyl ether extract of *Lotus peregrinus* treated group): Rats received diethyl ether extract (50 mg/kg b.wt) orally three times per weeks after complete induction of mammary cancer (at week 16) for six weeks at week 22.

2.9. Sampling:

2.9.1. Tissue samples:

About 0.5 gm of mammary tissue specimen was taken from each groups of rats after had been sacrificed at 22 weeks. The specimens were immediately removed and washed several times with saline and blotted between two damp filter papers, weighed and stored at -20°C for subsequent biochemical analyses.

2.9.1.1. Mammary tissue for biochemical analysis

Briefly, mammary tissues were cut, weighed and minced into small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH 7.4) to make 10 % homogenates. The homogenates were centrifuged at 6000 r.p.m for 15 minutes at 4°C then the resultant supernatant was used for the determination of the following parameters: L-MDA, CAT,

0.2 gm of mammary tissues were minced into small pieces homogenized with a glass homogenizer in 0.4 ml of 25% metaphosphoric acid (MPA) (ref. No.: 253-433-4, Sigma-Aldrich, Germany), then 1.4 ml of distilled water was added, mixed and incubated for 1 hour and centrifuged for 10 min at 3,000 r.p.m then the clean supernatant was removed and used for determination of GSH levels.

2.9.1.2. Mammary tissue for molecular gene expression

About 0.5 gm of mammary tissue put in eppendorf tubes and were immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for determination of caspase 9 and bax level.

2.10. Statistical analysis:

The results were expressed as mean ± SE using SPSS (13.0 software, 2009) program. The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparisons among the groups for testing the inter-grouping homogeneity. Values were considered statistically significant when $p<0.05$.

3. RESULTS

3.1. Chemical constituents of diethyl ether extract of Lotus peregrinus L.:

Clearly, this is the first report concerning diethyl ether extract of *Lotus peregrinus* L. chemical constituent and its biological effect on experimentally induced mammary cancer in rats ; median lethal dose of diethyl ether extract of *lotus* peregrinus L. showed LD_{50} =1000 mg/kg body weight as shown in Table (1) and Fig. (1). Thus, the dose used for injection is 1/20 of LD_{50}. Terpinoids of diethyl ether extract of *Lotus peregrinus* L. was determined using GC-MS technique.

Table (1) Median lethal dose of diethyl ether extract of *Lotus peregrinus* L.

Group	Dose (mg/kg)	Dose fold change	No. of animals	No. of dead animals	% mortality
G1	5	-	10	0	0
G2	50	10	10	0	0
G3	500	10	10	3	30
G4	1000	10	10	5	50
G5	2000	2	10	8	80

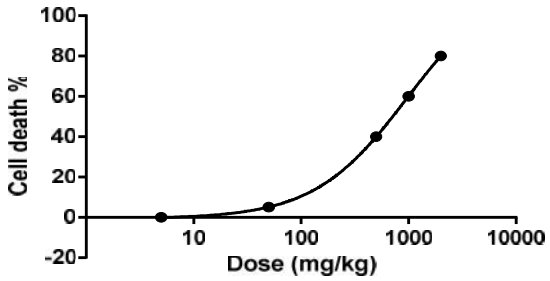

Fig. (1) Median lethal dose (LD_{50}) of Diethyl ether extract of *Lotus peregrinus* L.

The relative percentages of each component were calculated in Table (2) and illustrated in Fig. (2). Data revealed the presence of two triterpene: alpha-Amyrin and Lupeol in addition to one acyclic diterpene; Neophytadiene. In addition to hydrocarbon and sterols of diethyl ether of *Lotus peregrinus* L. was determined using GLC-MS technique. The relative percentages of each component were calculated in Table (3) and illustrated in Fig. (3). The highest percentage of hydrocarbon was that of Eicosane and with the highest percentage of sterol was that of Stigma-sterol. Qualitative and quantitative estimation for phenolic compounds of diethyl ether extract of *Lotus*

peregrinus L. were achieved using HPLC, where each compound was separated, identified using authentic pattern and determined its concentration as relative percent. The separated and identified phenolic compounds were Quercetin, Gallic acid; Pyrogallol, Chlorogenic, Caffeic, P-Coumaric, Ferulic acid, Ellagic acid, Salicylic and Cinnamic were detected as shown in Table (4) and Fig. (4).

3.2. Oxidative marker:

Chemically-induced mammary cancer rats (G2) had a significant (P≤0.05) increased MDA level as compared to untreated control group (G1) (Table 5). Prevention by diethyl ether extract fraction (G3) resulted in a significant decrease in MDA level. Moreover, post-treatment by diethyl ether extract fraction (G4) also led to a significant reduction in mammary tissues MDA level, as compared to that in the G2. Chemically-induced mammary cancer rats (G2) had a significant (P≤0.05) decrease Catalase activity and GSH level as compared to untreated control group (G1) (Table 4). Prevention by diethyl ether extract fraction (G3) resulted in a significant elevation in catalase activity and GSH level as compared to untreated mammary cancer group (G2).

Table 2 GLC analysis of hydrocarbon and sterols of *Lotus peregrinus* L. During the period of investigation (May, 2016).

P. No	Rt	Percentage (%)	No of C atom	Compound name
1	11.542	0.496	C14	N-Tetradecane
2	14.103	0.617	C16	N-Hexadecane
3	15.923	2.108	C18	N-Octadecane
4	17.677	1.092	C19	N-Nonadecane
5	19.147	8.027	C20	N-Eicosane
6	19.935	2.320	C21	N-Heneicosane
7	20.936	4.239	C22	N-Docosane
8	21.676	2.382	C23	N-Tricosane
9	22.954	7.456	C24	N-Tetracosane
10	23.864	3.256	C25	N-Pentacosane
11	24.800	5.737	C26	N-Hexacosane
12	25.584	2.536	C27	N-Heptacosane
13	26.582	7.372	C28	N-Octacosane
14	27.766	5.185	C29	N-Triacontane
Sterols				
16	30.797	3.146	C28	Compasterol
17	32.684	7.793	C29	Stigmasterol
18	34.092	5.334	C29	B-Sitosterol

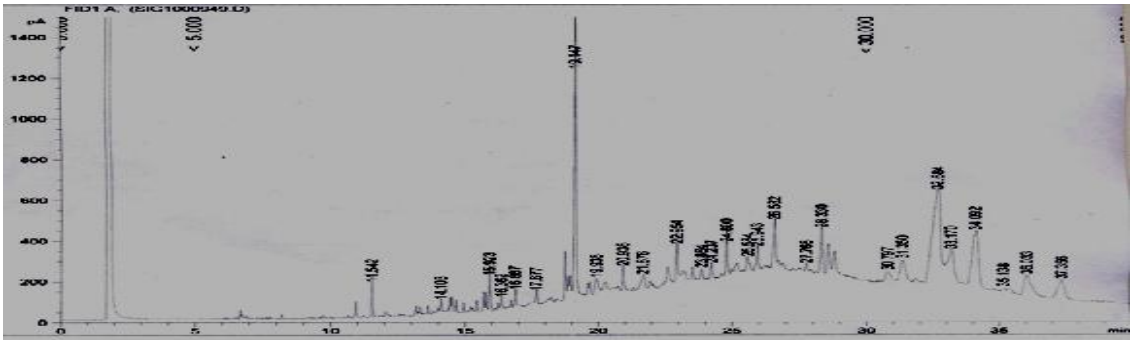

Fig. (2) GLC analysis of hydrocarbon and sterols of Lotus peregrinus L. During the period of investigation (May, 2016)

Table (3) GC-MS of terpinoids in diethyl ether extract of *Lotus peregrinus* L. during the period of investigation (2016).

P. No	RT	Area %	Molecular Formula	Compound name
1	32.641	6.15	C20H38	Neophytadiene (Acyclic diterpene)
6	50.454	0.75	C30H50O	Alpha-Amyrin (triterpene)
7	51.044	1.43	C30H50O	Lupeol (triterpene)

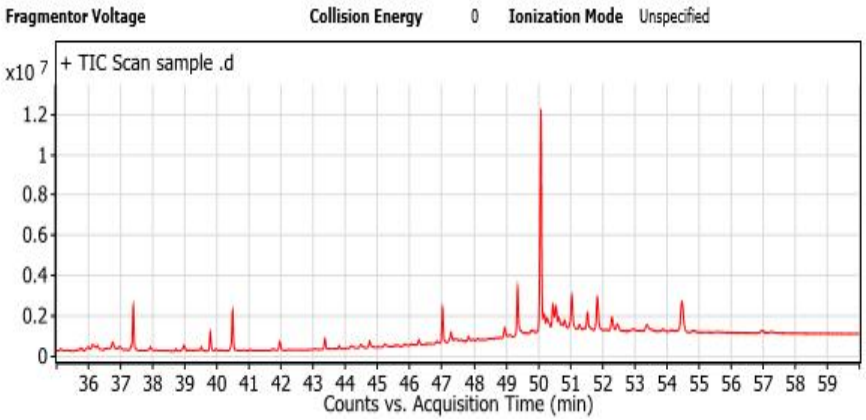

Fig. (3) GC-MS of terbinoids in diethyl ether extract of *Lotus peregrinus* L. during the period of investigation (2016).

Table (4) HPLC of phenolic compounds in diethyl ether extract of *Lotus peregrinus* L. during the period of investigation (May, 2016).

Peak no.	RT	Phenolic compound	Percentage (%)
1	3.654	Quercetin	19.51
2	7.650	Gallic	0.7447
3	8.640	Pyrogallol	0.6417
4	9.423	Chlorogenic	0.5952
5	11.109	Caffeic	0.9216
6	11.925	P-Coumaric	8.6379
7	12.621	Ferulic	7.2120
8	13.228	Ellagic	1.7145
9	14.242	Salycillic	18.6207
10	14.903	Cinnamic	13.9815

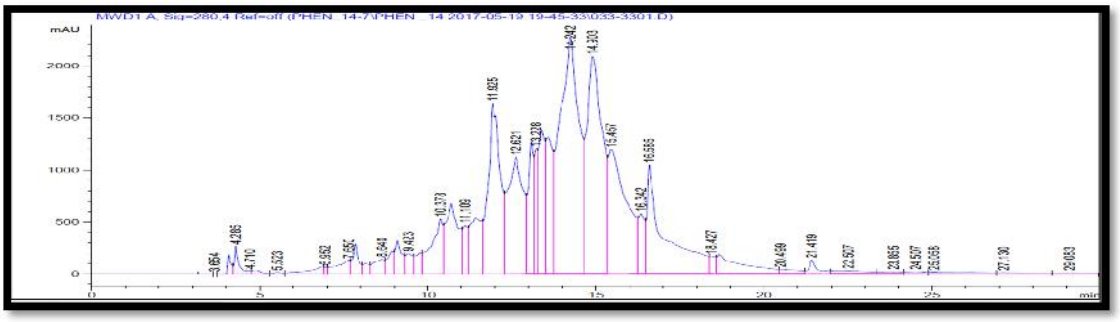

Fig. (4) HPLC of phenolic compounds in diethyl ether extract of *Lotus peregrinus* L. during the period of investigation (May, 2016)

Moreover, post-treatment by diethyl ether extract fraction (G4) also led to a significant increase in mammary tissues CAT activity and GSH level as compared to that in the G2.

3.3. Apoptotic marker (Bax and Caspase 9 gene expressions):

Our results revealed a significant (P≤0.05) down regulation of Bax and caspase 9 gene expression level in mammary gland of chemically-induced mammary cancer rats (G2) as compared to untreated control group (G1) (Table 6). Prevention by diethyl ether extract fraction (G3) resulted in a significant increase in Bax and caspase 9 expressions,

with highest expression in G3, as compared to untreated mammary cancer group (G2). Moreover, post-treatment by diethyl ether extract fraction (G4) also led to a significant elevated expression, as compared to that in the G2.

Table (5) Effect of diethyl ether extract of Lotus peregrinus L. administration on mammary tissue GSH, MDA concentration and CAT activities in chemically induced mammary cancer in wistar rats.

Exp. groups	L-MDA (nM/g tissue)	CAT(IU/g tissue)	GSH (IU/g tissue)
Group I:	20.53 ±1.72^{e}	5.14±0.32^{a}	26.42±1.52^{a}
Group Π :	57.11±2.13^{a}	1.08±0.07^{e}	7.46±0.43^{e}
Group III:	28.26 ±0.85^{d}	4.09±0.20^{b}	19.05±0.95^{b}
Group IV:	39.23±1.82^{c}	2.59±0.10^{d}	11.00±0.71^{d}

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table (6) Changes in relative expression of Bax and caspase 9 genes in mammary gland of chemically-induced mammary cancer rats following all treatments.

Exp. groups	Bax gene expression	Caspase 9 gene expression
Group I:	1.00±0.04 ^{a}	1.00±0.05 ^{a}
Group Π :	0.07±0.004 ^{f}	0.05±0.01 ^{e}
Group III:	0.70±0.03 ^{b}	0.64±0.05 ^{b}
Group IV:	0.27±0.01 ^{d}	0.28±0.03 ^{c}

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

4. DISCUSION

Mammary tumors induced by the administration of DMBA are morphologically and histologically similar to human mammary tumors and have been employed for investigating the chemo-preventive potential of medicinal plants and dietary agents (Kumaraguruparan et al., 2007). The present study investigated the effects of diethyl ether extract on the mammary cancer development, oxidative stress markers, and expression of bax and caspase 9 in mammary glands of DMBA-MPA induced rats. However, the effects of the diethyl ether extract of Lotus peregrinus L. on the expression of Bax and caspase 9 and levels of oxidative stress markers in 7,12 dimethyl benz(a)anthracene (DMBA)-Medroxyprogesterone- acetate induced experimental mammary cancer in wistar rats have not been previously reported. The diethyl ether extract of Lotus peregrinus L. Exert a potent antioxidant effect against cellular damage caused by reactive oxygen species. Our result supported by, Sameh, 2014 who reported that a significant decrease in mammary tissues GSH level was observed in DMBA induced mammary cancer in rats. On the other hand of our result, Ragab et al., 2013 reported that catalase activity and GSH in rats bearing mammary cancer was significantly increased. Beutlar and Gelbart, (1985) who demonstrated that antioxidant status has been suggested as a useful tool in estimating risk of oxidative damage induced carcinogenesis. Similarly with our result, Sameh , 2014 showed that a significant increase in mammary tissue (L-MDA) level were observed in DMBA induced mammary cancer in rats when compared with control group. Also, Al Shabrawy, 2015 mentioned that MDA level in tissue of rats showed that,

there was high significant increase in TBR (tumor bearing animal). In the same context, Sahin et al., 2011 mentioned that animals administered DMBA developed breast cancer, was associated with decreased expression of Bax and caspase 9 in mammary tissues. Similarly, with our result which shows that administration of diethyl ether extract of Lotus peregrinus L. was effective in inhibiting DMBA-MPA induced mammary tumors and modulating the expression of apoptosis associated proteins. This result may be due to present of a combination of bioactive constituents that have anticancer effect as terpenoides, sterols and different phenolic acid. This activity is confirmed by another study, Suwito et al., 2016 who mentioned that the IC50 of Lupeyl acetate, lupeol, and lupenone showed that anticancer activity of the prepared compounds following apoptosis mechanism. In addition to, Ewelyne et al., 2014 found that Cytotoxicity was only observed in fractions enriched with alpha and beta – amyrin and added that the resin and fractions enriched with α- and β-amyrin promoted cytotoxicity and apoptosis. Aziz et al., 2016 revealed that P.macrophylla leaf methanolic extract showed potent cytotoxicity towards the human breast cancer cells. The chemical constituent analysed by GC-MS reported 6 major compounds which are α-Cadinol, Neophytadiene, Palmitic acid, Linoleic acid and Oleic Acid. Additionally, many studies showed anticancer activity of phenolic acid that already separated from diethyl ether extract as; Ferulic acid ameliorated oxidative stress and improved the antioxidant status in ethanol-fed rats (Shivashankara et al., 2015). Aiyer and Gupta, 2010 reported that, ellagic may prevent mammary tumors by suppressing the levels of 17beta-estradiol E (2)-metabolizing enzymes during the early phase of E (2) carcinogenesis. This result support our revealed data, ellagic, gallic and ferulic acid were separated and identified from diethyl ether extracts. In addition to other separated and identified component (phenolic acid), Banerjee et al., 2015 suggested that, Gallic acid has a therapeutic potential against breast cancer. B-sitosterol supplementation to MDA-MB-231 cells induces 39% and 80% increases in the activities of caspases 8 and 9. There was also a 3-fold increase in the activity of caspase 3. It is concluded that β-sitosterol may induce apoptosis through the intrinsic pathway (Awad et al., 2003).

6. CONCULOSIONS

Clearly, this is the first report concerning investigation of chemical constituents of diethyl ether of Lotus *peregrinus* and its biochemical effect on chemically induced mammary cancer in rats. Diethyl ether was found to be potent against mammary cancer and promising natural agent. This anticancer effect of diethyl ether extract of *Lotus peregrinus* might be due to its synergistic action of bioactive compounds. The chemical composition of the diethyl ethyl extracts can be useful in the chemosystematics of this species.

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