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Phytochemical analysis and Antiproliferative activity of *lotus peregrinus* L. against MCF7 (breast carcinoma cell line)

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A B S T R A C T

The aim of this work was to investigate the phytochemical and anti-proliferative activity in vitro of the aerial parts of *lotus peregrinus* L. The preliminary phytochemical analysis of the aerial parts of *lotus peregrinus* L. (Family; Fabacea) revealed the presence of phenols, flavonoids, terpenoids, steroids, tannins and carbohydrates. Protein amino acids analysis showed the presence of 17 amino acids as protein amino acids. Aspartic acid (8.526 mg/g) and Glutamic acid (6.069 mg/g) represented as the major components of protein amino acids, respectively. Combined and free sugars analysis showed the presence of 12 free sugars. L-Rhaminose (21.9136) and Glucuronic (21.656 %) represented as the major free sugars, meanwhile the Xylose (29.425%); followed by Glucuronic (27.899%) represented as the major combined sugars. Twelve fatty acids were estimated; the highest percentage was that of Linoleic acid (18.489). Fourteen known hydrocarbon were found, the highest percentage was that of Eicosane (8.027%) in addition to three sterols; the highest percentage was that of Stigmasterol. two triterpene: alpha-Amyrin and lupeol in addition to one acyclic diterpene; Neophytadiene were detected. Anti-proliferative activity was carried out on the successive extracts (diethyl-ether, chloroform, ethyl acetate and ethanol 70% extracts) of *lotus peregrinus* L aerial parts to evaluate the Anti-proliferative properties. Diethyl ether was found to be potential anti-proliferative extract and promising natural agent. The chemical composition of the diethyl ethyl extracts can be useful in the chemosystematics of this species. Further studies will be needed to clarify the exact mechanism of *Lotus peregrinus* L. most active extracts as anti-cancer agent.

Key words: Anti-proliferative activity, *Lotus peregrinus* L., Phytochemical analysis

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1. INTRODUCTION

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). It accounts for nearly 21% of all cancers among women worldwide (Parkin and Fernandez, 2006). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body and these chemical substances are called phytochemicals. These phytochemicals were used to cure the disease in herbal and homeopathic medicines (Chitravadivu *et al*., 2009). 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). Flavones, particularly prenylated flavones, and triterpenes accompanied with sterols, anthraquinones , from genus *Flemingia Roxb.et Ait* plants have pharmacological effects including neuroprotection, anti-inflammation, anti-oxidation, cytotoxicity, and antimicrobial activities (Li *et al*., 2012). Family Fabaceae, the third-largest family in terms of number of species, with 630 genera and over 18,860 species and rich in medicinal plants. 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. The seeds of *Tamarindus indica* are used widely as a remedy for treating snake bites (Ushanandini *et al*., 2006). Leaves of *Sutherlandia frutescens* have antibacterial and antioxidant activity (Katerere and Eloff, 2005). The methanolic extract of *S. grandiflora* has antiviral and cytotoxic activities (Arthanari *et al*., 2012). Isoflavone lupiwighteone from Lotus pedunculated induces anticancer, apoptotic, and antiangiogenic activities (Ren *et al.*, 2015). (Ibrahim, 2016) showed that *Lotus halophilus* Successive extracts with different concentrations were tested against MCF7 (Breast carcinoma cell line) showing high cytotoxic value. Flavonoids have also been reported to induce the inhibition of cell growth in breast and prostate cancers, in vivo and in vitro (Sheppard *et al*., 1999; Limer and Speirs, 2004).

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; May (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 the extracts of Lotus peregrinus L.

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).

2.3. Preliminary Phytochemical Screening of the Aerial Parts of Lotus peregrinus L.

The concentrated residue from the gradient solvent extracts of the plant material was used to detect the secondary plant metabolites, this include testing for tannins (Balbaa, 1986), testing for sterols and terpenes (Tiwari et al., 2011), testing of alkaloids (Harborne, 1973; Trease and Evans, 1996), testing for flavonoids and phenolic compounds (Edeoga et al., 2005), testing for carbohydrates (Balbaa, 1986), testing for saponins (Tiwari et al., 2011).

2.4. Amino Acid Contents

The investigation of protein amino acids was carried out qualitatively and quantitatively by using LKB alpha plus amino acid analyzer

S433/SYKAM according to the method described by (Steven et al., 1989).

2.5. Carbohydrate Contents

2.5.1. Free sugars:

Extraction of free sugars described by (Chaplin & Kennedy, 1994).

HPLC of free sugars (Nagel, 1992): The sugars were determined by using High Performance Liquid Chromatography (HPLC) 1050, whereas the extracted sugars were injected.

2.5.2. Combined sugars:

Hydrolysis of combined sugars described by (Chaplin and Kennedy, 1994).

2.6.Hydrocarbon and sterols contents

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).

2.7. Fatty acid contents

The investigation of Hydrocarbon and sterols were carried out qualitatively and quantitatively by using Gas Liquid Chromatography. The relative proportions of each individual compound were estimated as the ratio of the partial areas to the total area as mentioned by (Fryer et al., 1960; Nelson et al., 1969 and Farag et al., 1986).

2.8 Terpenoid contents

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.

2.9 Anti-proliferative Assay: Cell viability determination by MTT assay

The anti-proliferative activity of the diethyl ether , chloroform, ethyl acetate and 70 % ethanol were evaluated by using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Molecular probes, Eugene, Oregon, USA; Cat.no.V-13154)]which is a yellow tetrazole, is reduced to purple formazan in living cells. A solubilisation solution (usually dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a coloured solution. The MTT method allows one to measure the number of viable cells in a given sample as the percentage of viable cells present in the control sample. The principle of this method is based on cellular reduction of MTT to a blue formazan product by mitochondrial dehydrogenases of viable cells. The intensity of the blue color formed by this procedure is proportional to cell viability (Bianco et al., 2012).

3. RESULTS

Pharmacopoeial constants of *Lotus Peregrinus* L. showed in table (1), it was noticed that (Table 2 & Fig. 1) the maximum percentage of free sugars was L-Rhamnose (21.9136), followed by Glucuronic (21.656 %), meanwhile the minimum percentage was the maximum percentage of hydrolyzed combined sugars (Table 3 & Fig. 2) was Xylose (29.425%); followed by Glucuronic (27.899%), meanwhile the minimum percentage was sorbitol (0.040 %). It can be concluded that the major protein amino acids were aspartic acid (8.526 mg/gm), followed by glutamic acid (6.069 mg/gm) while methionine (0.108 mg/gm) was the minor protein amino acid showed in table (4) and illustrated in (Fig 3 & 4). Our data revealed the presence of fourteen known hydrocarbon;

the highest percentage was that of Eicosane (8.027%) and three sterols; the highest percentage was that of Stigmasterol (7.793 %) shown in table (5) and Fig. (5). Our data revealed the presence of twelve fatty acids were estimated, eight saturated fatty acids beside four unsaturated fatty acid; the highest percentage was that of Linolenic acid; this data showed in table (6) and Fig. (6). Our data revealed the presence of two triterpene: alpha-Amyrin and Lupeol in addition to one acyclic diterpene; Neophytadiene; data represents in table (7) and Fig. (7).

Table (1) Certain pharmacopoeial constants of *Lotus Peregrinus* L. during the period of investigation (May 2016)

Constant	Percentage (%)
Water content	31.62
Inorganic matter (total ash)	8.53
Organic matter	94.47
Acid insoluble ash	3.273
Acid soluble ash	5.257
Water insoluble ash	5.412
Water soluble ash	3.118
Crude fibers	21.64

Table (2) HPLC of free sugars of Lotus Peregrinus L. during the period of investigation (May 2016).

S .No	RT	Sugar	Percentage (%)
1	4.984	Glucuronic	21.6562
2	5.690	Stachyose	18.7929
3	6.638	Galacturonic	3.5107
4	7.042	Sucrose	3.3287
5	7.346	Glucose	9.0710
6	8.348	Xylose	11.9333
7	10.567	Galactose	6.4813
8	12.148	L-Rhamnose	21.9136
9	13.767	Mannose	1.0528
10	14.230	Arabinose	0.9842
11	15.758	Fructose	0.4470
12	16.927	Mannitol	0.3817

Where RT: Retention Time

Table (3) HPLC of combined sugars of *Lotus Peregrinus* L. during the period of investigation (May 2016).

S .No	RT	Sugar	Percentage (%)
1	2.623	Inulin	0.3945
2	4.877	Glucuronic	27.8899
3	5.588	Galacturonic	3.8431
4	6.181	Sucrose	2.2733
5	7.383	Glucose	10.7297
6	8.346	Xylose	29.4250
7	10.291	Arabinose	20.6200
8	11.853	Fructose	2.4234
9	14.172	Manitol	2.3605
10	20.288	Sorbitol	0.0406

Where RT: Retention Time

Table (4) The separated protein amino acids of *Lotus peregrinus* L. using Amino Acid Analyzer. During the period of investigation (May 2016).

S .No	RT	Protein amino acids	Conc (mg/gm)	Percentage (%)
1	9.45	Aspartic acid	8.526	17.9
2	11.57	Threonine	2.516	5.3
3	12.46	Serine	3.249	6.8
4	15.02	Glutamic acid	6.069	12.8
5	21.11	Glycine	2.966	6.2
6	22.23	Alanine	3.457	7.3
7	23.97	Valine	2.06	4.3
8	26.51	Methionine	0.108	0.2
9	28.29	Isoleucine	1.464	3.1
10	29.26	Leucine	3.166	6.7
11	31.49	Tyrosine	1.044	2.2
12	32.68	Phenyl Alanine	2.836	6.0
13	35.17	Histidine	2.745	5.8
14	38.63	Lysine	3.132	6.6
15	40.59	Ammonia	2.757	5.8
16	42.47	Arginine	5.795	3.0

Where: RT = Retention time

S .No	RT	Protein amino acids	Conc (mg/gm)
1	16.840	Prolin	3.643

The MTT assay was performed to detect the inhibitory effect of diethyl ether, chloroform, ethyl acetate and total ethanoic of *Lotus peregrinus* L. on human breast cancer cell line, MCF7. MTT assay results exhibited significant dose dependent anti-proliferative activity for diethyl ether, chloroform, ethyl acetate and 70 % ethanoic of *Lotus peregrinus* L. on MCF7; with IC50 values of 26.91, 62.73, 37.76 and 109.8 % respectively.

Table (5) GLC analysis of hydrocarbon and sterols of Lotus peregrinus L. During the period of investigation (May 2016).

S. 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		Compasterol
17	32.684	7.793		Stigmasterol
18	34.092	5.334		B-Sitosterol

Table (6) GLC analysis of fatty acids of *Lotus peregrinus* L. during the period of investigation (May 2016).

S .No	RT	Percentage (%)	No of C atom	Compound Name
1	9.466	2.099	C12	Lauric acid
2	10.534	2.127	C13	Tridecanoic acid
3	11.923	4.733	C14	Myristic acid
4	13.728	2.242	C15	Pentadecanoic acid
5	15.970	1.286	C16(1)	Palmitoleic acid
6	18.400	9.241	C18(1)	Oliec acid
7	19.441	18.489	C18(2)	Linoleic acid
8	20.687	10.030	C18(3)	Linolenic acid
9	22.565	1.873	C20	Arashidic acid
10	23.778	3.614	C22	Behenic acid
11	26.447	2.930	C24	Lignoceric acid
12	29.039	2.993	C26	Hexacosanoic acid

Table (7) GC-MS of terpinoids in diethyl ether extract of *Lotus peregrinus* L. during the period of investigation (2016).

S .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)

As compared to vehicle (DMSO)-treated MCF7 cells are graphically illustrated in (Figure 8-11).

4. DISCUSION

Our study results showed that diethyl ether extract of aerial part of *Lotus peregrinus* L. had the most potent cytotoxicity followed by ethyl acetate extract against MCF7 with mean IC_{50} values of 26.91 and 37.76 µg/ml respectively. Here we present qualitative and quantitative study of the composition of diethyl ether extract (hydrocarbon, sterols, fatty acid and terepenoids) from the aerial parts of *Lotus peregrinus* L. grown in wadi habis in marsa matrouh (Egypt).

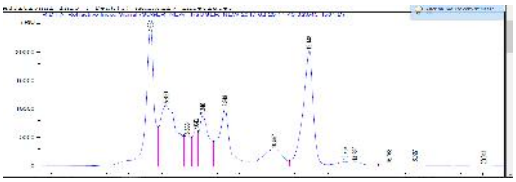

Fig. (1) HPLC of free sugars of *Lotus Peregrinus* L during the period of investigation (May 2016)

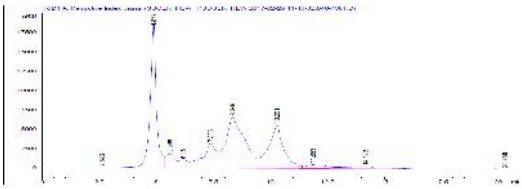

Fig. (2) HPLC of combined sugars of *Lotus Peregrinus* L. during the period of investigation (May 2016).

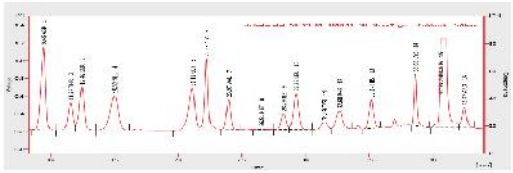

Fig. (3) The separated hydrolyzed protein amino acids of *Lotus peregrinus* L. using amino Acid Analyzer. During the period of investigation (May 2016).

Fig. (4) The separated hydrolyzed protein amino acid (Prolin) of *Lotus peregrinus* L. using amino Acid Analyzer. During the period of investigation (May, 2016)

Fig. (5) GLC analysis of hydrocarbon and sterols of *Lotus peregrinus* L. During the period of investigation (May 2016).

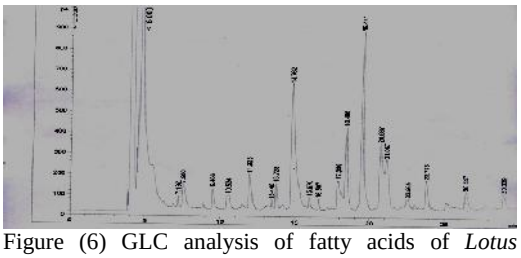

Figure (6) GLC analysis of fatty acids of *Lotus peregrinus* L. during the period of investigation (May 2016).

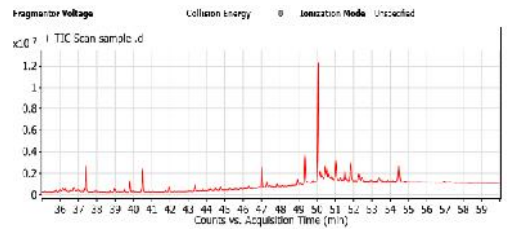

Fig. (7) GC-MS of terpinoids in diethyl ether extract of *Lotus peregrinus* L. during the period of investigation (2016).

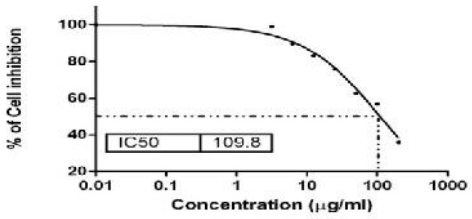

Fig. (8) Percentage of survival fraction against concentration (µg/ml) of the 70% ethanoic extract of *Lotus peregrinus* of breast carcinoma cell line.

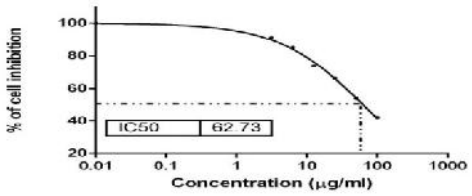

Fig. (9) Percentage of survival fraction against concentration (µg/ml) of the chloroform extract of *Lotus peregrinus* of breast carcinoma cell line.

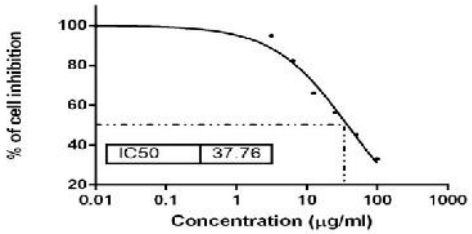

Fig. (10) Percentage of survival fraction against concentration (µg/ml) of the ethyl acetate extract of *Lotus peregrinus* of breast carcinoma cell line.

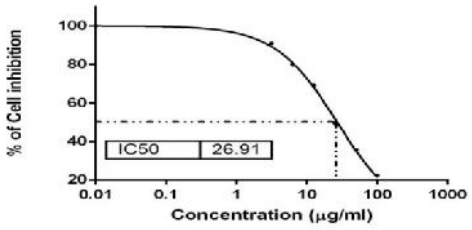

Fig. (11) Percentage of survival fraction against concentration (µg/ml) of the diethyl ether extract of *Lotus peregrinus* of breast carcinoma cell line

These results may be attributed to the presence of certain terpenoids in the diethyl ether extract. Our result supported by other previous studies , which have a reported cytotoxic effect (Suwito *et al*., 2016; Aziz *et al*., 2016; Ewelyne *et al*., 2014). Similarly, (Ibrahim, 2016) showed that *Lotus halophilus* successive extracts with different concentrations were tested against MCF7 showing high cytotoxic value. In addition to ethyl acetate which contains phenolic compounds which have been found to possess a protective effect against cancer by their effect on signal transduction in cell proliferation and angiogenesis (Fatemeh and Khosro, 2013).

Wang *et al.* (2016) reported that, lupeol inhibited the migration and invasion of human breast cancer MDA-MB-231 cells in a dose- dependent manner in vitro. Similarly, Suwito *et al.* (2016) showed that the IC50 of Lupeyl acetate, lupeol, and lupenone showed that anticancer activity of the prepared compounds following apoptosis mechanism. Ewelyne *et al.* (2014) found that Cytotoxicity was only observed in fractions enriched with alpha and beta - amyrin. Altogether, 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 significant and potent cytotoxicity towards the human breast cancer cells. The chemical constituent analysed by GC-MS reported detected 6 major compounds which are α-Cadinol, Neophytadiene, Palmitic acid, Linoleic acid and Oleic Acid.

The chemical composition of the diethyl ethyl extracts can be useful in the chemosystematics of this species; Ash content is useful in assessing the quality of grading the plant and also gives an idea of the amount of minerals present in the sample (Michael and David, 2002). Rhamnose is commonly bound to other sugars in nature. It is a common glycone component of glycosides from many plants. Rhamnose is also a component of the outer cell membrane of acid-fast bacteria in the Mycobacterium genus, which includes the organism that causes tuberculosis (David et al., 2005). Xylitol prevents a shift of the bacterial community towards a more cariogenic micro-flora in oral environment (Trahan, 1995). Oral ingestion of xylitol causes a smaller rise in plasma glucose and insulin concentrations than does the ingestion of glucose in healthy men and diabetics (Amo *et al.*, 2011). Aspartic acid is a non-essential

amino acid, which means that it is manufactured from other amino acids in the liver; also it is essential for purine, pyrimidine, asparagine and inositol synthesis. Aspartic acid has been used as mineral salts such as magnesium aspartate or potassium aspartate to help improve energy production in exercising muscles. (Akram *et al.*, 2011). 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). Linoleic acid is one of the shortest-chained Omega- 6 fatty acids (C18:2) and is categorized as an essential fatty acid because the human body cannot synthesize it. Omega-6 fatty acids are a family of pro-inflammatory and anti-inflammatory polyunsaturated fatty acids (Scorletti and Byrne, 2013). Linoleic acid is converted to gamma-linolenic acid (GLA) in the body. GLA may actually reduce inflammation. Some studies showed that, taking gamma linolenic acid (GLA) for 6 months or more may reduce symptoms of nerve pain in people with diabetic neuropathy, people who have good blood sugar control may find GLA more effective than those with poor blood sugar control (De Lorgeril and Salen, 2012). These previous reports can support the helpful role of linoleic acid in the studied biological effects of the plants.

5. CONCULOSIONS

Clearly, this is the first report concerning cytotoxicity efficacy of *Lotus peregrinus* L. extracts. Diethyl ether was found to be potential anti-proliferative extract and promising natural agent. The cytotoxic activity of *Lotus peregrinus* L. 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. Further studies will be needed to clarify the exact mechanism of *Lotus peregrinus* L. most active extracts as anti-cancer agent

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