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IN VITRO ANTICANCER (HELA), ANTI-INFLAMMATORY, BRINE SHRIMP LETHALITY ASSAY AND GC-MS ANALYSIS OF WHOLE PLANT *ARCEUTHOBIUM OXYCEDRI* (DWARF MISTLETOE) N-HEXANE FRACTION

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ABSTRACT

Mistletoe has been used for anticancer treatment in different parts of the world. Most of the chemotherapeutic drug molecules are isolated from mistletoe. The present study aims to comprehensively examined for the first-time whole extract and different fractions of *Arceuthobium oxycedri* (drawf mistletoe) belonged to Ziarat Juniper Forest of Balochistan, Pakistan. The whole plant extract and its different fractions were investigated for some biological activities such as: anticancer by MTT assay on HeLa cell line, Brine Shrimp Lethality by β-hatching technique and Anti-inflammatory by chemiluminescence technique in oxidative burst assay. The structural elucidation was done by GC-MS and a total of 21 compounds were identified. The whole plant *A. oxycedriI,* n-hexane fraction (WAOHF), exhibited 69.3% mortality against cervical cancer cell line (HeLa) with IC_{50} value of 11.4 ± 0.7. The brine shrimp lethality activity in WAOHF showed 53.3% mortality at highest concentration. The anti-inflammatory activity in whole plant *A. oxycedri* methanolic extract (WAOME), WAOHF and whole plant *A. oxycedri* aqueous fraction (WAOAF) were inactive. The obtained results revealed that the WAOHF exhibited significant anticancer against cervical cancer cell line (HeLa), which can be considered as an alternative therapeutic anticancer drug in future with least side effects.

Keywords: *Arceuthobium oxycedri*; Anticancer; Anti-inflammatory; Brine Shrimp Lethality; n-hexane fraction

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INTRODUCTION

Mistletoe is a semi parasitic herb which germinates on deciduous trees found around the world. Mistletoe belongs to the Viscaceae family it grows on deciduous trees like apple, tea, pine, lime, mango, pear, larch and approximately 1500 species have been identified (Grieve, 1994; Becker, 1986; Ergün, 2021). In Pakistan two species of *Arceuthobium* have been reported, in which *Arceuthobium oxycedri* (dwarf mistletoe) infected Juniper (*Juniperus excelsa* M. Bieb.) of Ziarat forest (Sarangzai *et al*., 2010). The average size of Asia, Africa and Europe mistletoe is in between 2-15 cm, it is dioecious plant with small stem and small leaves with sessile flowers of small sheets (Ciesla *et al*., 2002). It inhibits the growth of host plant growth by photoassimilates outflows which reduce its drought resistance and enhance fungal and bacterial invasions (Hawksworth, 1996). Mistletoe is an herbal drug, used in a treatment against cancer (Büssing, 2000). Iscador® (*Viscum album* L.) commercial name of European mistletoe, after different clinical trials, it has been suggested as a postoperative treatment for cervical, colon, lungs and breast carcinomas (Grossarth-Maticek, 2001). In cancer treatment the extract of mistletoe has been utilizing as a complementary drug. In numerous investigation mistletoe extracts hypodermically, trails have shown productive immunodulatory effects (Kienle and Kiene, 2003). Mistletoe has two biologically active compounds, Viscotoxin and Lectins are known as cytotoxic agents (Rostock *et al*., 2005). In Turkey dwarf mistletoe used as an effective remedy for different diseases like, upper respiratory inflammatory and infectious disorder, hypotensive remedy for gastrointestinal disorders (Akkol *et al*., 2010). The (+)-catechin compound isolated from ethyl acetate (EtOAc) fraction of *A. oxycedri*, which possess remarkable antioxidant property (Orhan *et al*., 2019). The main purpose of this research was to find out for the first time anti-inflammatory, anticancer (HeLa) and cytotoxicity of different fractions of *Arceuthobium oxycedri* (dwarf

mistletoe) from Ziarat Juniper Forest of Balochistan, Pakistan.

MATERIALS AND METHODS

Plant material: *Arceuthobium oxycedri* sample were collected from Ziarat Juniper forest, Balochistan, Pakistan. Plant identified by taxonomist Prof. Dr. Rasool Bakhsh Tareen and Dr. Shazia Saeed, Botany Department, University of Balochistan, Quetta, Pakistan.

Plant extraction: The whole plant *A. oxycedri* was first washed with tap water and then dried the plant in shade for a month at room temperature. 2 Kg grinded fine powder of whole plant was soaked in methanol (5 Liter) stirred it well and left the mixture for seven successive days at room temperature. The methanolic supernatant of whole plant was filtered by Whatman filter paper, and then filtrate was evaporated by rotary evaporator at 40^0C under lower pressure to yield crude extract. The semisolid methanolic crude extract of *A. oxycedri* (WAOME) was 193.28 gm. 30 gm of crude extract was preserved to examine anti-inflammatory, Brine Shrimp Lethality Assay (BSLA) and anticancer (HeLa). The remaining methanolic crude extract was further fractionated with n-hexane and aqueous (Achakzai and Anwar, 2016).

Fractionation of crude extract: In separatory funnel the crude extract with two solvents such as n-hexane and aqueous were added. The two layers were formed in separatory funnel after shaken thoroughly. Separated the aqueous and n-hexane layers and then vaporized individual solvent to form n-hexane and aqueous fraction by rotary evaporator. 7.8 gm whole plant *A. oxycedri* n-hexane fraction (WAOHF) and 138.1 gm whole plant *A. oxycedri* aqueous fraction (WAOAF) obtained, which was examined for anti-inflammatory, Brine Shrimp Lethality Assay (BSLA) and anticancer (HeLa) (Achakzai and Anwar, 2016).

HeLa cell line: The modified Dulbecco's Eagle medium, along fetal bovine serum 10% was utilized for HeLa cell line culture, incubated that at 37^0C in 5% carbon dioxide incubator. HeLa cells were assembled when the confluency developed completely on 96 well flat where 8000 cells were plated per well. Added 50 µg/ml fractions after 24 hours completed and further incubated that for next 48 hours. All the fractions were removed after the incubation time period. In each well added 0.5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution and incubated at 37^0C for 3 hours. Micro-plate reader (Spectra Max plus, Molecular Device, CA, USA) was used to measure the absorbance at 570 nm. Doxorubicin drug was used as a standard in this assay. The % inhibition was calculated by following formula

$$\% \text{ inhibition} = \frac{\text{mean of O. D. test compound} - \text{ mean of O. D. of negative control}}{\text{means of O. D. of positive control} - \text{ means of O. D. of negative control}} \times 100$$

The calculation for IC_{50}, 20 mM extract and fractions and stock solution was diluted into 50 µM working solution and to obtain less than 50% inhibition, working solution was diluted in further serial dilutions. EZ-fit3 software was used to calculate IC_{50} (Scudiero *et al*., 1988).

Anti-inflammatory assay: Oxidative burst assay, chemiluminescence technique was used a 25 µl of extract and fractions with 25µl diluted solution of whole blood $HBSS^{++}$ were incubated at 37^0C for 15 min. After incubation step, plated them into 96 well plates. The blank wells contained $HBSS^{++}$ while the control wells contained cells and $HBSS^{++}$. Added 25µl serum – opsonized zymosan and 25µl luminol in each well. ROS level was measured in luminometer. Standard drug (Ibuprofen) used in this assay, with IC_{50} 11.2 ± 1.9 µg/mol (Helfand *et al*., 1982).

Brine shrimp lethality assay: In hatching tray, disperse 50 mg eggs of brine shrimp in a brine solution that were incubated at 23^0C. In 20 ml solvent dissolved 20 mg extract and fractions. Transferred the solution of 5, 50, 500µl into 3 vials, the solution was left overnight for evaporation. 10 larvae placed in each vial with the help of Pasteur pipette, then added 5ml seawater and incubated the mixture at (25-27^0C) under illumination for 24 hours. In other vials for reference was added cytotoxic drug along solvent for negative and positive control. LD_{50} was determined by Finney computer program (Alves *et al*., 2000; Kivçak *et al*., 2002; Carballo *et al*., 2002).

Gas Chromatography mass spectrometry (GC-MS) analysis: For quantification and identification of *A. oxycedri* compounds, Injected 2µl *A. oxycedri* extract and fractions in gas chromatography (mod. 6890N Network GC, Agilent Technology Palo Alto, CA) which further coupled with mass spectrometer (mod. 5973 Network) and mass selective detector (Agilent Technology Palo Alto, CA). GC-MS HP-5MS column (30m in length, 0.25µm film width, 0.25 mm interior diameter), its split-splitless injector splited the ratio 30:1 at 250^0C. The oven temperature arranged such as, 70^0C for 3 min, 6°C/min – 180^0C for 5 min and then 6^0C/min – 280^0C for 10 min, 8^0C/min – 290^0C for 20 min. The transfer line of MSD was arranged at 250^0C, the temperature of MSD quardopole was 150^0C, ionization was at 230^0C; mass spectra was 70 eV where the scan achievement was in between 35 and 300m/z. The components of *A. oxycedri*

extract and fractions were identified by libraries like WILEY and NIST02 (El-Wakil *et al*., 2015).

RESULTS

Anticancer activity (HeLa cell line): As far world's different mistletoes efficacy regarding anticancer activities was very effective and results proved that they can be very effective herbs in cancer treatments, so it was obvious to find out the same

The efficiency regarding the anticancer activity of *A. oxycedri* (dwarf mistletoe) collected from juniper forest of Balochistan, Pakistan on HeLa cell line was studied. Doxorubicin was taken as standard drug which IC_{50} was 0.9 ± 0.1, and the results of whole plant *A. oxycedri* n-hexane fraction (WAOHF) exhibited 69.3% cytotoxicity against cervical cancer (HeLa) cell line with IC_{50} value of 11.4 ± 0.7, which was more effective than standard drug. Whole plant *A. oxycedri* methanolic extract (WAOME) exhibited 21.5% inhibition which was below the standard drug, whole plant *A. oxycedri* aqueous fraction (WAOAF) shown inactive activity against HeLa cell line. The results of whole plant extract and fractions of *A. oxycedri* anticancer activities (HeLa) cell line shown in Table 1.

Anti-inflammatory: *A. oxycedri* (dwarf mistletoe) was analyzed for the first time as anti-inflammatory activity by chemiluminescence technique in oxidative burst assay, with three graded samples concentration (10, 50 and 250 µg/ml) and Ibuprofen was taken as standard drug which IC_{50} was 11.2 ± 1.9 µg/ml. The anti-inflammatory activity in whole plant *A. oxycedri* methanolic extract (WAOME), whole plant *A. oxycedri* n-hexane fraction (WAOHF) and whole plant *A. oxycedri* aqueous fraction (WAOAF) were inactive. The results of whole plant extract and fractions anti-inflammatory activities have shown in Table 2. The results of the present study regarding anti-inflammatory activity found in disagreement reported by Kuttan *et al*. (2017) where different mistletoe species like *V. trilobatum* and *V. capitallatum* showed significant anti-inflammatory activity with (P<0.001).

Brine shrimp lethality: *A. oxycedri* (dwarf mistletoe) of Ziarat juniper forest of Balochistan methanolic extract was firstly screened for its cytotoxicity at brine shrimp and results were 100% at 1000 µg/ml, 96% at 100 µg/ml and 84% at 10 µg/ml, which was very significant (Zaidi *et al*., 2006). It was obvious to analyze its further sub fractions for cytotoxicity in brine shrimps. The present study was done on further fractions of *A. oxycedri*, which was n-hexane fraction and aqueous fraction. Three graded sample concentration (10, 100 and 1000 µg/ml) were used. Etoposide was taken as standard drug with 46.66% mortality. The brine shrimp lethality activity in whole plant *A. oxycedri* n-hexane fraction (WAOHF) showed 53.3% mortality at highest concentration (1000 µg/ml) on brine shrimps. The results of whole plant extract and fractions brine shrimp lethal activity have shown in (Table 3-5).

GC-MS: Whole plant *A. oxycedri* n-hexane fraction showed significant anticancer and cytotoxicity, for that reason the structural elucidation was carried out by GC-MS. Twenty-one compounds were identified in whole plant *A. oxycedri* n-hexane fraction (WAOHF). The molecular formula, mass, structure and retention time are present in (Table 6). While the mass spectra interpretation of these 21 compounds are present in (Table 7).

Table 1: Anticancer Assay (HeLa cell line) of extract and all fractions of whole plant *A. oxycedri*

S No	Extract/ Fractions/ Std. Drug	Conc. (µg/ ml)	% Inhibition/ Stimulation	IC_{50} ± S.D
1	WAOME	50	21.5	Inactive
2	WAOHF	50	69.3	11.4±0.7
3	WAOAF	50	0.2	Inactive
4	Doxorubicin	50	101.2	0.9±0.1

Table 2: Anti-inflammatory activities of extract and all fractions of whole plant *A. oxycedri*

S No	Extract/ Fractions/ Std. Drug	Conc. (µg/ml)	% Inhibition/ Stimulation	IC_{50} ± S.D µg/ml
1	WAOME	250,50,10	-	Inactive
2	WAOHF	250,50,10	-	Inactive
3	WAOAF	250,50,10	-	Inactive
4	Ibuprofen			11.2 ± 1.9

Table 3: Brine Shrimp Lethality Assay in whole plant *A. oxycedri* Methanol extract (WAOME).

Dose (µg/ml)	No of Shrimps	No of Survivors	%Mortality	STD.Drug	%Mortality
10	30	28	6.66		
100	30	28	6.66	Etoposide	46.66
1000	30	25	16.66		

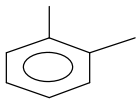
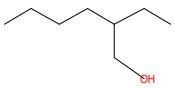
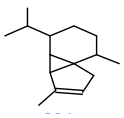
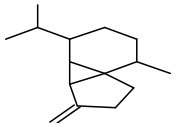
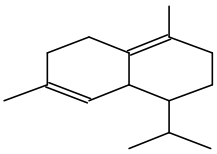
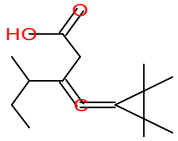
Table 4: Brine Shrimp Lethality Assay in whole plant *A. oxycedri* n-Hexane fraction (WAOHF).

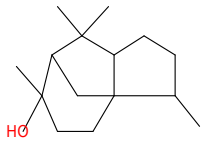
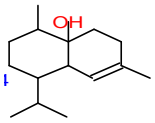
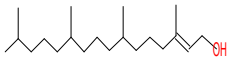
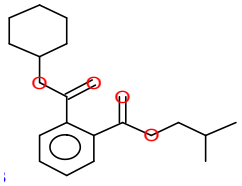
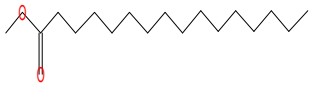
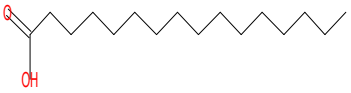
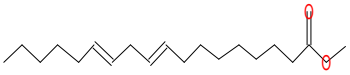
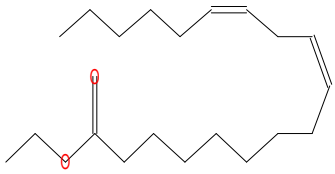
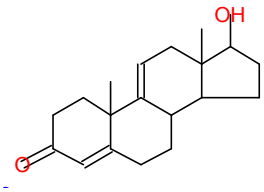
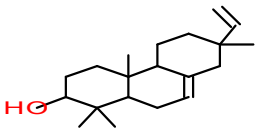
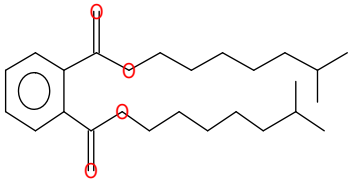
Dose (µg/ml)	No of Shrimps	No of Survivors	%Mortality	STD.Drug	%Mortality
10	30	29	3.33		
100	30	27	10	Etoposide	46.66
1000	30	14	53.3		

Table 5: Brine Shrimp Lethality Assay in whole plant *A. oxycedri* Aqueous fraction (WAOAF).

Dose (µg/ml)	No. of Shrimps	No. of Survivors	% Mortality	STD. Drug.	% Mortality
10	30	28	6.66		
100	30	28	6.66	Etoposide	46.66
1000	30	27	10		

Table 6: Molecular mass, Molecular formula and RT of whole plant *A. oxycedri* n-Hexane fraction (WAOHF).

Compound	RT	Molecular Formula	Molecular Mass	Structure
1	5.61	C8H10	106	
2	8.34	C8H18O	130	
3	14.72	C15H24	204	
4	15.28	C15H24	204	
5	15.58	C15H24	204	
6	15.76	C15H24O2	236	

7	16.68	C15H26O	222	
8	16.88	C15H26O	222	
9	19.46	C20H40O	296	
10	19.98	C18H24O4	304	
11	20.02	C17H34O2	270	
12	21.88	C16H32O2	256	
13	25.74	C19H34O2	294	
14	28.43	C20H36O2	308	
15	34.52	C19H26O2	286	
16	36.76	C20H32O	288	
17	38.98	C24H38O4	390	

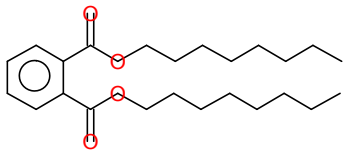
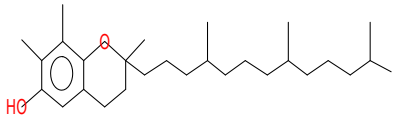
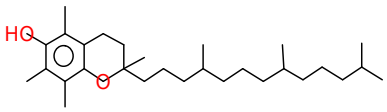
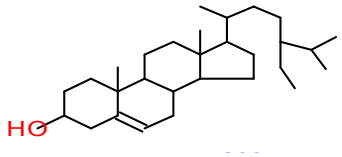
18	38.98	C24H38O4	390	
19	43.97	C28H48O2	416	
20	44.90	C29H50O2	430	
21	47.66	C29H50O	414	

Table 7. Whole plant *A. oxycedri* n-hexane fraction (WAOHF) compounds (1-5) mass spectra.

Compound	m/z (% relative abundance)
1	106(M^+) 91 (999), 106(632). 105(263), 77(108), 51(81), 92(79,) 39(75), 79(64), 65(63), 103(57)
2	130(M^+) 57(999), 41(337), 43(300), 70(260), 83(259), 55(258), 56(222), 29(172), 98(130), 69(122)
3	204(M^+) 161(999), 105(976), 119(933), 41(336), 81(320), 91(292), 120(286), 93(259), 55(234), 204(204)
4	204(M^+) 161(999), 105(343), 91(304), 120(258) 41(244), 119(210), 81(189), 79(175), 93(165) 55(160)
5	204(M^+) 161(999), 134(578), 119 (501), 204(474), 105(469), 81(255) 159(248), 91(245), 41(228) 93(207)
6	236(M^+) 41(999), 161(713), 57(675), 73(537), 119(504), 221(472), 105(470), 133(458), 91(452), 77(343)
7	222(M^+) 95(999), 150(811), 151(683), 43(597), 41(562), 81(415), 69(360), 55(295), 107(270), 93(258)
8	222(M^+) 119(999), 41(937), 43(919), 161(909), 93(634), 59(614), 105(586), 55(528), 81(485), 79(420)
9	296(M^+) 81(999), 82(986), 43(965), 95(962), 123(892), 55(852), 41(811), 57(811), 71(748), 68(728)
10	304(M^+) 149(999), 150 (95), 223(76), 167(54), 57 (50), 104(28), 55(27), 41(23), 76(16), 67(15)
11	270(M^+) 74(999), 87(720), 43(325), 55(310), 41(228), 143(208), 75(188), 57(183), 69(132), 227(110)
12	256(M^+) 60(999), 73(980), 57(840), 43(817), 55(767), 41(574), 129(435), 71(373), 69(351), 83(267)
13	294(M^+) 67(999), 81(775), 55(742), 41(717), 95(557), 82(509), 68(473), 54(447), 96(398), 79(354)
14	308(M^+) 67(999), 81(831), 55(669), 95(586), 41(518), 68(476), 82(459), 54(455) 69(374) 96(362)
15	286(M^+) 286(999), 253(556), 271(342) 91(255), 287(232), 41(210), 57(189), 244(178), 77(168), 105(168)
16	288(M^+) 255(999), 288(975), 105(600), 119(570), 133(443), 148(435), 91(413), 107(405), 93(368), 44(353)
17	390(M^+) 149(999), 167(350), 57(341), 70(264), 41(225), 71(224), 55(218), 43(200), 150(107), 83(100)
18	390(M^+) 149(999), 167(540), 57(442), 71(358), 70(281), 43(273), 279(219), 41(211), 113(207), 55(165)
19	416(M^+) 151(999), 416(312), 150(216), 152(137), 191(132), 417(98), 43(55), 55(44), 57(44), 69(40)
20	430(M^+) 165(999), 430(494), 164(332), 43(178), 431(155), 166(122), 57(104), 205(91), 55(75), 121(71)
21	414(M^+) 43(999), 55(667), 57(563), 41(507), 81(481), 95(449), 107(440), 69(362), 414(354), 145(321)

DISCUSSION

Different species of dwarf mistletoes found in different regions of the world were examined for pharmaceutical purpose because the local community utilize this plant as an effective remedy for different diseases most commonly for cough, cold, gastrointestinal, respiratory treatment etc. (Livon, 1913). Over the last decade different species of mistletoes have also been tested for their anticancer activity, several studies got positive results and from the last several years in different region of the world, such as common mistletoe (*Viscum album*) and dwarf mistletoe (*Arceuthobium americanum*) have been used for the cancer treatments and their constituent had shown anti-tumor activity (Selawry *et al*., 1959; Maldacker, 2006; Hoessli and Ahmad, 2008; Hajtó *et al*., 2011; Strüh *et al*., 2013; Loef and Walach, 2020). In *V. album* the toxic protein found which is used for cancer therapy (Luther *et al*., 1987). The *A. americanum* also contain a type of toxic protein with low concentration, which can also be used for the cancer treatment (Samuelsson, 1969). The results of *A. oxycedri* n-hexane fraction has also shown positive result for HeLa cell line, 69.3% cytotoxicity with IC_{50} value of 11.4 ± 0.7, which can also be used for the cancer treatment.

Inflammation is basically immune respond of tissues to the various stimuli (Fontes *et al*., 2015). Macrophages are inflammatory cells which activate by inflammatory process, by the release of inflammatory mediator such as chemokines, cytokines and nitric oxide (de la Fuente *et al*., 2012). Whole plant *A. oxycedri* extract and all fractions were inactive none of fraction exhibited anti-inflammatory activity. The obtained results showed that the studied plant *A. oxycedri* (dwarf mistletoe) used during the present study do not possess anti-inflammatory activity. The obtained finding disagreed with other studies such as: different fractions of *Tapinanthus bangwensis* (also called African mistletoe) were examined for anti-inflammatory activity on wistar albino rat, in which ethyl acetate and butanol fractions showed highly significant anti-inflammatory activity (Patrick-Iwuanyanwu *et al*., 2010). Also disagree with another study in which Turkish *A. oxycedri* n-butanol and EtOAc extract and subfractions exhibited significant activity as Anti-inflammatory (Akkol *et al*., 2010).

Bioactive compounds of natural origin, their cytotoxic effects are usually checked by brine shrimp lethality test. BSLT is mostly use for initial screening for anticancer bioactivity (Pisutthanan *et al*., 2013; Hamid *et al*., 2011). *A. oxycedri* only methanolic extract was first screened for cytotoxicity at high concentration 1000 µg/ml with 100% mortality (Zaidi *et al*., 2006). Whole plant *A. oxycedri* n-hexane fraction (WAOHF) showed 53.3% mortality at highest concentration. The cytotoxicity of n-hexane fraction supports the use of *A. oxycedri* in future as potential medicine, and this initial screening support the idea to further find out anticancer activity by studying on different cancer cell lines.

Conclusion: A total 21 compounds were identified in whole plant *A. oxycedri* n-hexane fraction (WAOHF) which also exhibited inhibition on (HeLa) cell line with 69.3% mortality along with IC_{50} value of 11.4 ± 0.7. The brine shrimp lethality activity in WAOHF showed 53.3% mortality at highest concentration. The obtained results of the present study revealed that the WAOHF exhibited significant anticancer against cervical cancer cell line (HeLa) and cytotoxic activity. It is concluded from the present study that the n-hexane extract of *A. oxycedri* can be considered as an alternative to the therapeutic anticancer drug in future with least side effects as other mistletoes species which are using in European countries for the cancer treatment.

Conflicts of interest: The authors declared that there aren't conflicts of interest.

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