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EXTRACT OF 1:5 MIXTURE OF FIVE MOROCCAN MEDICINAL PLANTS HAS CYTOTOXIC EFFECT ON SOME HUMAN CANCER CELL LINES

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ABSTRACT

Traditional medicines have long been used by the Moroccan people. Several medicinal recipes prepared from a mixture of plants are often used by traditional medicinal practitioners for the treatment of many diseases including cancer. However, little is known for their scientific information especially their anticancer effects. In this study, one traditional used Moroccan herbal remedy for treatment of hemorrhoids and cancer therapy (cervical cancer, skin cancer) was investigated for its cytotoxic effect against cancer cell lines. Aqueous herbal distillate of mixture in equal quantity of five medicinal plants was evaluated for its *in vitro* cytotoxicity, using the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, against two human cancer cell lines; cervix adenocarcinoma (HeLa) and breast cancer cell line (MCF7). The mixture of plants exhibited potent cytotoxicity with an IC50 of 60 µg/ml for Hela cells and 122 µg/ml for MCF7 cells. Furthermore, the mixture was evaluated *in vitro* for antioxidant potential with the spectrophotometric method based on the reduction of the stable DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical. The IC50 value was calculated in order to evaluate the antioxidant efficiency of plants mixture. The Aqueous herbal distillate of mixture exhibited moderate antioxidant activity 62.76 %, with an IC50 value of 96.8 µg/ml compared to the IC50 value of 4.73 µg/ml as shown by the reference antioxidant Trolox. In conclusion, this herbal remedy that was used to treat cancer patients has effective cytotoxic effect on cancer cells *in vitro*, and further studies of the active extracts are necessary for chemical characterization of the active compounds and more extensive biological evaluations.

Keywords: Cytotoxic effect, aqueous herbal distillate, mixture of medicinal plants, cancer cells, antioxydant potential.

INTRODUCTION

Mortality that results from the common forms of cancer is still unacceptably high. Despite many therapeutic advances in the understanding of the processes in carcinogenesis, overall mortality statistics are unlikely to change until there is a reorientation of the concepts for the use of natural products as new anticancer agents. Natural or semisynthetic compounds may be used to block, reverse, or prevent the development of invasive cancers. This is the reason that is considerable scientific and commercial interest in discovering new antioxidant and anticancer agents from natural product sources [1]. The potential of using natural products as anticancer agents was recognized in the 1950s by the U.S. National Cancer Institute (NCI) and since then several studies have given valuable contributions to the discovery of new naturally occurring anticancer agents. Attempts are underway to work out the therapeutic and anti-neoplastic properties of medicinal plants [2] and, consequently,

herbal medicines have received much attention as substitute anticancer drugs. Plant kingdom is a potential source of chemical constituents with antitumor and cytotoxic activities owing to their enormous propensity, which synthesize a variety of structurally diverse bioactive compounds [3]. In recent years, there has been a great deal of attention and discoveries in exploiting plant kingdom for pharmaceutical application and the interest in plants as a source of potential therapeutic agents, particularly as anticancer agents [4]. Uncontrolled proliferation is a universal property of tumor cells. Investigation of the cellular growth control mechanisms has contributed to the understanding of carcinogenesis and identification of compounds with specific antitumoral activities [5].Thus, cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antitumoral properties for future studies [6]. Many modern drugs with plant origin have been discovered following folklore claims of their efficacy combined with the extensive ethnobotanical knowledge of local peoples. There are two main strategies for the selection of plants species in anticancer drug discovery: random screening and ethnomedical knowledge. The second approach includes plants used in organize traditional medical systems like herbalism and folklore [7]. As Morocco is rich in natural resources, plants have played a major role and appreciated for treating illness in Moroccan traditional medicines for many years [8,9,10]. Lots of cancer Moroccan patients are still using traditional medicine as an alternative medicine for their own diseases [11]. The medicinal plants can also reduce or minimize the toxic side effect of chemotherapy and radiation treatment by reinforcing their cancer killing action and including cancer. Unfortunately, little information about the efficacies of theses herbal remedies has been known and information regarding the bioactive compounds and the therapeutic activities of them is still lacking. In recent years, most of research performed today, in Morocco, focuses on the contribution of development of new natural drugs to treat cancer, as well as others infection [12].

This study has aimed to investigate the cytotoxic and antioxidant effect of mixture composed in the same proportion of five selected Moroccan medicinal plants. HeLa and MCF7 cancer cell lines were used because these two cancers have high incidence in Moroccan population.

The selection of this mixture was made on the basis of his reputation as folk medicines in treatment of cancer of the cervix, skin and the treatment of hemorrhoids. The mixture is composed of five medicinal plants: *Cannabis sativa* L; *Lavandula pedunculata*, *Cistus ladanider* L; *Dittrichia viscosa* (L.) Ait; *Nerium oleander* L. The Table 1 shows the ethnobotanical data of the investigated plant species, including botanical names, local names, ethnomedical uses, as well as the plant parts employed in this study. The results from this study will be beneficial for evaluation of this mixture for his efficacy as anticancer and antioxidant agent and valorization of Moroccan medicinal plant as alternative medicine.

MATERIALS AND METHODS

Plant material: Plant materials were collected. The determination of the botanical names of the plants was done in collaboration with Dr. M. Fennane, an expert botanist from the scientific National Institute, Rabat. Voucher specimens of each plant have been deposited in the herbarium of Scientific Institute, University Mohammed V–Rabat–Morocco.

Preparation of aqueous herbal distillate: The five plants are used in the same proportions. The components of each plant are flowers, leaves, fruit and stems. For 100 g of mixture, 20 g of each plant cut into small pieces were placed in distillation apparatus and hydrodistilled was used. The distillate is recovered, concentrated and kept at-20 ° C.

Cytotoxic activity

Cell Culture: Cancerous cells were cultured in DMEM media (Sigma, USA) supplemented with 10% heat-inactivated FBS and 1% antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin).The cells were grown at 37◦C in a humidified incubator set at 5% CO2. Cells were subcultured after they formed a monolayer on the flask. The cells were detached by treating them with trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10 minutes and then by adding complete medium to inhibit the reaction.

In Vitro Cytotoxicity Assay: The antiproliferative potential of the mixture extract was evaluated against adenocarcinoma cervical cancer (HeLa) cell line and Human breast cancer cell line (MCF7), by the MTT assay [13]. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product. Briefly, cells were seeded in a 96-multiwell plates (8. 10^3 cells/well), and treated with various concentrations of extract (3.9–250 µg/ml) for 72 h. Mitomycine, Etoposid and Carboplatin were used as positive control. After 72 h of incubation, 10µL of solution MTT (5 mg/ml) was added to each well and incubated at 37°C for 4 h. After 72 h of incubation, 10µL of solution MTT (5 mg/ml) was added to each well and incubated at 37°C for 4 h. The medium was removed and the insoluble formazan crystal was dissolved in with 150 µl of DMSO. Finally, the

absorbance was read with an ELISA reader (Thermo Scientific, Paris, France) at 550 nm. Experiments were performed in quadruplicate (n = 4) and data were expressed as means ± SDs. IC50 (inhibitory concentration 50%) and SD (standard deviation for 95% confidence) were determined by interpolation from the viability curves of cells versus plant extract concentrations. Cell viability was determined by the MTT assay (n = 4). Viability curves: Percentage viability = absorbance of test wells/absorbance of control wells) × 100) plotted against the concentration of extract.

Antioxidant activity

Free Radical Scavenging Activity: The free radical scavenging activity of the plants mixture was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) [14]. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of plants mixture (final concentrations were 31.25, 62.5, 125, 500 and 1000µg/ml, respectively) or methanol (control).and was allowed to stand at room temperature for 30 min, and then absorbance was read at 517 nm against blank samples. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation:

% RSA = $[(A_{DPPH} - A_{Extr})/ A_{DPPH}] \times 100$. Where A_{DPPH} is the absorbance value of the DPPH blank sample, and A_{Extr} is the absorbance value of the test solution. A_{Extr} was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank.

Statistical analysis: Results of the research are expressed as mean ± S.D (standard deviation) and were tested for statistical significance by one-way ANOVA. Differences were considered statistically significant at the $P < 0.05$ level. The IC50 values were obtained by nonlinear regression using the SPSS 13.0 program.

RESULTS AND DISCUSSION

Cytotoxic activity

MTT test: Plants are promising source of anticancer chemotherapeutic agents, the use of plant derived natural compounds as part of herbal preparations and alternative sources of medicaments continues to play major roles in the general wellness of people all over the world [15]. The search for anticancer agents that may inhibit cancer development is becoming an important objective for scientists. In this study we have explored the cytotoxic effect of plants mixture, and the choice of this mixture is justified by its anticancer properties in traditional medicine. Plants mixture was subjected to cytotoxicity assays against HeLa and MCF-7 cell lines by MTT assay, this method is important to select plant extracts with potential anticancer properties. The mixture was found to show dose dependent cytotoxicity against HeLa and MCF-7 cell lines between the concentration ranges of 3.9 – 250 µg/ml. The plants showed a significant cytotoxicity against HeLa cell lines. The IC50 values were also confirmed that mixture showed cytotoxicity against this tested cell line more than that of MCF-7 cells (HeLa IC50 = 60 µg/ml, MCF-7 IC50= 122 µg/ml) ($P<0.01$). (Fig. 1-2) The results of the study extend previous finding that the mixture of the plants tested contains numerous biologically active compounds and some of these have been frequently used in folk medicine for their anticancer properties. Phytochemical studies have identified active components in the *Nerium oleander*, such as cardiac glycosides like oleandrin, oleandrigenin, digoxin, digitonin, digitoxigenin, nerizoside, neritaloside, odoroside [16], it is demonstrated that *Nerium oleander* chemical compounds are having significant cytotoxic activity, in fact oleandrin, digoxin or ouabain produce apoptosis in prostate tumor cells and that this effect is mediated through inhibition of Na+, K+ -ATPase [17,18]. Cannabinoids, the active components of *Cannabis sativa* are used as potential antitumoral agents on the basis of experiments performed both in cultured cells and in animal models of cancer [19] and have been known to exert palliative effects in oncology [20,21]. *Lavandula pedunculata* has a stimulatory effect on appetite; the species is used to treat Anorexia which is an important risk factor for morbidity and mortality in cancer [22]. Morocco has great potential in plants and traditional therapy botanical data showed that *Dittrichia viscosa is* traditionally used to treat cancer [23]. Flavonoids and sesquiterpene compounds isolated from *Dittrichia viscosa* showed a selective antiproliferative activity and apoptosis inducing effects against MCF7 cells [24]. *Cistus* species are known for their antimicrobial effect [25], but its cytotoxic effect was never studied. The chemical composition of Cistus species reveals the presence of flavonoids and phenolic acids derivatives, known in by their pharmacologic and therapeutic effect [26].

The results obtained in this study indicate that the mixture of all species cited was shown to induce significant inhibitory activity against human cancer cell lines tested. We adopted the criteria of the American National Cancer Institute to consider a crude extract promising for further purification based on the IC50 values lower than 30 µg/ml in order to discover and develop potential anticancer natural compounds [27], the mixture exerts a pronounced

dose-dependent inhibitory effect. The cytotoxicity is clearly identified against cancer cell lines; we could infer that the cytotoxic activity of plants mixture is the synergistic effect of their compositions and to the presence in the plants of active products that could probably have highly anti-growth effects. It would be an attractive medicinal preparation to further explore in depth its anticancer properties, it is important to examine the effects of mixture species against specific cancer cell lines to identify preliminary candidates for alternative cancer therapeutics.

Antioxidant activity

Free Radical Scavenging Assay: The mixture was evaluated for its radical scavenging activities by means of the DPPH assays. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 518 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition [28]. Based on the results of DPPH-radical assay the antioxydant activity of the Aqueous herbal distillate of mixture was concentration-dependent compared with that of positive control, (Trolox), the mixture tested exhibited moderately DPPH radical-scavenging activities. At 500 and 1000 µg/ml, the mixture inhibited more than 50.33 and 62.76 % respectively of the DPPH radical with an IC50 of 96.8µg/ml.

For a long time, plant-derived antioxidants have been used to reduce the level of oxidative stress within the human bodies. Hydroxyl radicals are the most reactive of all the reduced forms of dioxygen and are believed to initial cell damage and induce cellular senescence. Recently, knowledge and application of potential antioxidant activities in reducing oxidative stresses *in vitro* and *in vivo* have prompted many investigators to search for potent natural antioxidants from various plant sources [29,30]. The reducing capacity of a natural compound or extract may serve as a significant indicator of its potential antioxidant activity [31].There is a highly positive relationship between the natural compounds of many plant species and antioxidant activity, because of the scavenging ability of their hydroxyl groups [32]. It is also reported that plants naturals compounds are effective hydrogen donors, making them very good antioxidants [33] considered a promising therapeutic approach as they may be playing neuroprotective (preventing apoptosis) and neurodegenerative roles.

In this study, the reducing power of the plants mixture increased with increasing its concentration, indicating that some compounds in the Aqueous herbal distillate of mixture were electron donors and could also react with free radicals to convert them into more stable products and to terminate radical chain reactions. It would be interesting to fractionate the plants mixture to identify the active compounds.

CONCLUSION

This work reveals that the Moroccan flora can be an interesting source of new anticancer and antioxidant drugs, but further studies should be adopted to fractionate the mixture plants to identify the active compounds and to determine the exact mechanism of action of the constituents possessing potential cytotoxic activity.

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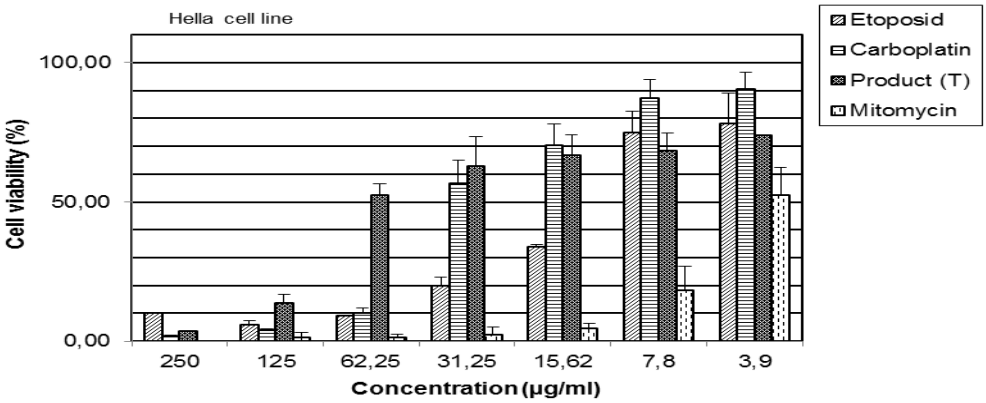

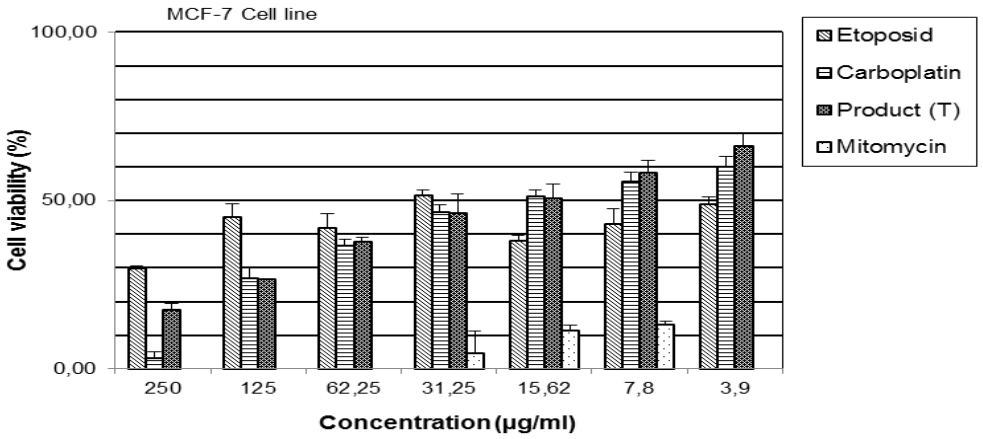

Fig. 1: Percentage cell viability curve of plants mixture against HeLa (a) and (b) MCF-7 cell lines.

Cell viability was plotted via the concentration and all samples were run in quadruplicate (n=4). The percent viable cells were calculated in comparison to untreated cells taken as 100%. Values were expressed as mean ± SD ($P<0.05$).

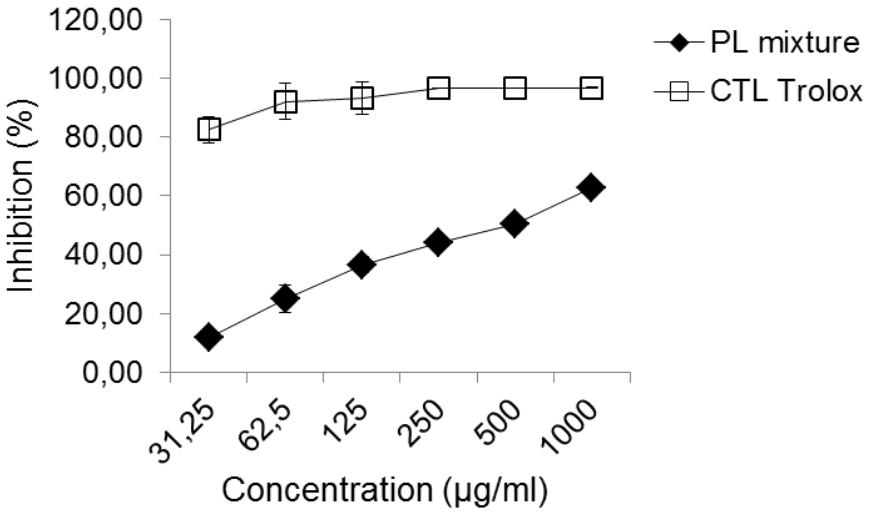

Fig. 2: Free-radical scavenging activity of plants mixture extract measured using the DPPH assay. PL mixture: Plants mixture. Values are means ± standard deviation with respect to positive control (Trolox). Bars having different letters indicate significant statistical difference ($P<0.05$).

Table 1. Ethnobotanical data and some reported pharmacological activities of plants species used in this study.

Botanical names (Family)	Local names	Common name	Plant parts used	Traditional uses	Pharmacological activities
Lavandula pedunculata (Labiatae)	halhâl	Lavande pédunculée	Flowers, leaves	Anxiety, insomnia, anorexia [34], Tonic, cough, bronchitis, asthma, rheumatism and stomach ache [35]	Antifungal activity [36] Acethylcholine esterase inhibition and antioxydant activity [37]
Cistus ladanider L (Cistaceae)	Targla, ftâh	Ciste ladanifére	Roots	Digestive disorders, gastric acidity [35]	Antimicrobial activity [37] Antioxydant activity [38] Herbicidal activity [39]
Dittrichia viscosa (L) Ait (Asteraceae)	Mâgrâmân, terhalâ	Aunée visqueuse	Leaves	Skin diseases, treats cutaneous abcesses, wound healing, Tuberculosis, bronchial infections [35]	Inflammatory effects [40] Antimicrobial activity [41] Antifungal activity [42] Antitumoral activity [43]
Nerium oleander L (Apocynaceae)	ddeflà	Laurier rose	Leaves, roots and stems	Colds, headaches, diseases of the uterus, burns, tumors, scabies, vermin, hair loss, dental pain, asthma and rheumatism [35]	Antifungal and antibacterial activity [44] Immunomodulating activity [45] Antileukemic and Anticancer activity [46]
Cannabis sativa L (Cannabaceae)	l.kif, l. qanneb	Chauvre indien	Roots	Sedative effect, tranquilizer and anesthetic [35]	Anticancer activity [47] Antimicrobial activity [48] Antineoplastic activity [49]

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