The Effect of *Gnaphalium oxyphyllum* var. *natalie* on Cancer and Blood Cells

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Abstract: *Gnaphalium oxyphyllum* DC is a medicinal plant whose common uses by Mexican people include the treatment of cancer. The toxicity of the aqueous and organic fractions as well as the aqueous decoction of *G. oxyphyllum* var. *natalie* F. J. Espinosa were assessed on three human cancer cell lines as well as in blood cells in healthy human lymphocyte cultures. Cytotoxic activity was assessed by the Sulforhodamine B method on HeLa (human cervical carcinoma), T47D (human breast carcinoma) and 22Rv1 (human prostate carcinoma cancer). Colchicine was used as positive control. The decoction was also tested on lymphocytes from healthy donors through the mitotic index as biomarker. We used whole blood for these cultures and estimated the effect of the extract on platelets, leukocytes and erythrocytes. The aqueous decoction was cytotoxic (ED₅₀ < 20 μg/mL) on the three cancer cell lines. The mitotic index in the exposed lymphocyte cultures did not significantly differ from the control nor the blood counts showed any difference between the experimental and control cultures. These results prove that the toxic effect of the aqueous decoction of *G. oxyphyllum* var. *natalie* is specific for cancer cell lines.

Key words: *Gnaphalium oxyphyllum*, cancer cell lines, cytotoxicity, lymphocyte culture, mitotic index.

1. Introduction

In Mexico, there are about 30,000 species [1] of higher plants and about 3,000 of them are medicinal plants [2]. Plants more frequently used in Mexican traditional medicine are known by the name of “gordolobo” [3] and belong to genus *Gnaphalium* L., so if you are looking for this plant in a medicinal herb stand, they can give you *G. americanum* Mill., *G. conoideum* Lam., *G. inornatum* DC., *G. liebmannii* Klatt, *G. oxyphyllum* DC., *G. semiamplexicaule* DC., or *G. viscosum* Kunth, [2, 4, 5].

Since prehispanic times, members of this genus have been called as “Tzonpotonic” (fly hair) in Nahuatl language [6]. Their main usage in Mexican traditional medicine is in the treatment of various respiratory diseases such as gripe, fever, asthma, throat ache, bronchitis and cough (7, 2). Plants from the genus *Gnaphalium* are also used in some other Latin American countries to cure skin infection, as an anti-inflammatory agent, as well as anti-rheumatic [8, 9]. Species from this genus that have been employed to treat breast cancer are *G. arenarium* L., *G. conoideum* K., and *G. luteoalbum* L. meanwhile *G. obtusifolium* L., *G. palycephalum* Michx., and *G. spicatum* Mill. are used to treat tumors [10].

Several studies have been performed to evaluate the therapeutically potential of this genus. For example, it has been reported that chemical compounds isolated from hexane and methanol extracts from *G. oxyphyllum* var. *oxyphyllum*, *G. liebmanni* var. *monticola* (McVaugh) D. L. Nash and *G. viscosum* have antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* [7]. Moreover, the methanolic extract from *G. oxyphyllum* inhibited in 42.15 % the growth of *Entamoeba histolytica* at a concentration of 150 μg/mL [11].

*G. americanum* also showed antibacterial activity...
on *S. aureus*, *S. pneumoniae* and *S. pyogenes* [4]. *G. stramineum* Kunth is a Colombian medicinal plant with anti-inflammatory activity and has been suggested that its biological effects may be due to the presence of a combination of caffeoylquinic acid derivatives and flavonol glycosides [9]. The ethyl acetate fraction of the methanol extract from *G. sylvaticum* displayed moderate inhibitory activity (57.2%) against the HIV-1 reverse transcriptase [12]. Based on the folk usage of plants from this genus, we chose *G. oxyphyllum*. In Mexico the varieties *oxyphyllum* and *natalie* are found [13]. Their flowers and steams are used in Mexican traditional medicine as tea that is drank to treat diabetes [14], cough, respiratory ailments, asthma (2), headache [15], and stomach ache [16]. They are also used in the treatment of gastric ulcer, against intestinal parasites, wounds, fever, dropsy, lumbago and cancer [17]. Unfortunately the ethnobotanical data only identify the plant material up to the species level. In this study the taxonomic identification of the plant material collected reached the variety level resulting *G. oxyphyllum* var. *natalie* and we studied its effect on cancer and healthy blood cell cultures. The plants of this species grow between 2,250 to 3,050 m altitude, in tropical and temperate areas, in pine and oak forests. There are herbaceous annual or perennial plants of 6 to 60 cm height with capitate inflorescences disposed in panicles or corymbs. Each capitulum with 3 to 5 series of imbricate greenish-white to reddish-white bracts, that becomes green-yellowish to brown-yellowish in herbarium specimens. Peripheral flowers are female, filiform, 3 to 4-lobulate, and the central are bisexual, tubular and 5-lobulate; all are white to yellow and bear no ligula [13].

2. Material and Methods

2.1 Plant Material

*G. oxyphyllum* var. *natalie* was collected in Apan, Hidalgo, Mexico, and identified at the herbarium of the Universidad Autónoma Metropolitana-Iztapalapa. A voucher is kept in this University with number UAMIZ 65464 for future reference. The stems and leaves from young plants were obtained and cleaned in order to remove dust and other debris. Then they were placed into paper bags and dried at room temperature. Finally they were ground to obtain small pieces to increase the contact area to the solvent systems employed.

2.2 Cancer Cell Lines

Breast cancer T47D, prostate cancer 22Rv1 and cervix cancer HeLa cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA).

2.3 Blood Cells

Heparinized blood samples were taken from healthy nonsmoking subjects, 23-year-old on average, who had not taken any medication for at least 30 days before the blood donation. Written consent was obtained from each donator.

2.4 Preparation of Extracts and Fractions

The steam and leaves of ground dried young plants were macerated with CH$_2$Cl$_2$:CH$_3$OH (2:1) by a 3 day period. After this time, the liquid was obtained and the marc was macerated with methanol for 3 days. The methanol extract was obtained. The CH$_2$Cl$_2$: MeOH extract was concentrated until 1/3 of its original volume and after adding water, a precipitate with the characteristic smell of the original plant, was formed; then this precipitate was separated.

The mixture CH$_2$Cl$_2$: MeOH: H$_2$O was macerated with CH$_2$Cl$_2$ in order to obtain the aqueous and organic phases.

The methanolic extract was also concentrated until 1/3 of its original volume and it was processed as the CH$_2$Cl$_2$: MeOH extract in order to obtain the organic and aqueous phases.

2.5 Preparation of the Decoction

Decoction from *G. oxyphyllum* var. *natalie* was
obtained by refluxing the ground dried plant in water for 4 hours. The liquid was filtered and the water was eliminated using a hot water bath.

2.6 Evaluation of Cytotoxic Activity

T47D (human breast cancer), 22Rv1 (human prostate cancer) and HeLa (human cervix cancer) cell lines were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine (In Vitro, Mexico), 10% heat inactivated fetal calf serum (In vitro, Mexico) and an antibiotic mixture of streptomycin (50 µg/mL) and penicillin (50 UI/mL) (In vitro, Mexico). All cancer cell lines were cultured at 37 °C in an atmosphere of 5 % CO2/95 % air (100 % humidity). At 70-80 % confluence cells were detached from the culture flask by treatment with 0.05% trypsin-EDTA (In vitro, Mexico). The cells were counted and their viability determined using trypan-blue (Sigma) exclusion dye on a hemocytometer (Boecco, Germany). After counting, dilutions were made to obtain the required cell density (T47D = 1.0 x 10^5 cel/mL; HeLa = 5.0 x 10^4 cel/mL, 22Rv1 = 8.1 x 10^4 cel/mL). In each case, 96 well tissue culture plates were used. An aliquot of 100 µL of cell suspension was added to each well. 100 µL of the complete medium was added to cell-free wells. The microtite plates containing cells and medium were preincubated for approximately 24 hours at 37 °C in an atmosphere of 5 % CO2/95 % air (100 % humidity) incubator to allow stabilization prior the addition of the samples.

Then the cells were treated with 100 µL of four different concentrations (for triplicate) of extracts dissolved in DMSO (Sigma, Mexico) (dimethyl sulfoxide) to a final concentration of 0.5 % in the culture medium and incubated for 48 hours and fixed by addition of 50 µL of cold 50 % (w/v) trichloroacetic acid-TCA- final concentration of 10 % in the culture medium and incubated for 1 hour at 4 °C.

The supernatant was discarded and the plates were washed five times with tap water and air dried. The cells were stained with 50 µL/well of sulforhodamine B-SRB- (Sigma, USA) solution (0.4 %, w/v, in acetic acid) for 30 min at room temperature. Unbound SRB was removed by washing five times with 1 % acetic acid and the plates were air-dried; bound SRB stain was solubilized with 200 µL of 10 mM unbuffered Tris base (Sigma, Mexico), pH 10. The plates were placed on a shaker for 5 minutes and the absorbance was read on a spectrophotometric plate reader at 490 nm (BIO-RAD model 450, USA). Cell population density at time zero was measured from reference plate of cells fixed with TCA just prior to sample addition and processed as described above [18]. The absorption values obtained with each of the treatment procedures were averaged, and the averaged value obtained with the zero day control subtracted. Cell survival was obtained as (T/C) x 100 where T and C represent the mean absorbance of cells treated with sample and vehicle control (DMSO), respectively. Results were expressed as the dose that inhibits 50 % control growth after the incubation period (ED50).The ED50 values were calculated by regression analysis (percent survival versus log concentration). Colchicine (Sigma, USA) was used as positive control. According to the standards of the NCI (National Cancer Institute), ED50 values of ≤ 20 µg/mL for extracts and ≤ 4 µg/mL for pure compounds are considered active [19]. Three independent experiments were made.

2.7 Mitotic Index and Blood Cell Count

Aliquots of 0.3 mL whole blood samples from four healthy donors were placed into plastic sterile tubes (Nunc) containing 2.5 mL of McCoy 5a modified medium (Microlab, Mexico) added with 4% Phyttohemaglutinin M (Microlab, Mexico) and 0.4 % antibiotic solution (In vitro, Mexico). After 24 h of incubation at 37 °C, 5 groups of three cultures each were formed and exposed to the following final concentrations of the aqueous extract of G. oxyphyllum var. natalie: 0, 2, 10, 50 and 500 µg/mL.
The final DMSO concentration in these cultures was 0.1 %, and this DMSO concentration was used in another set of cultures that served as negative control. All cultures were then incubated during 48 h at 37 °C.

After the exposition period, aliquots of 500 µL were taken to count leukocytes, erythrocytes and platelets using an automatic blood counter cell (KX-21-N).

The remaining cells in cultures were treated with 60 µL of Colcemid (Microlab, Mexico) for 1 hour at 37 °C, to arrest T lymphocytes in metaphase. Cells were separated from the culture medium by centrifugation, exposed to a hypotonic solution of 0.4% KCl (J. T. Baker, Mexico) for 20 min and fixed with Camoy’s solution (acetic acid:methanol, 3:1; J. T. Baker, Mexico). Slides were stained with 10 % Giemsa (Merck, Mexico). The mitotic index was calculated from a total of 6000 cells (dividing + non dividing cells) for each group and donor [20].

2.8 Statistical Analyses

The ED₅₀ values were obtained with the Excell program. Statistical test used to compare the effects of the aqueous decoction on blood healthy cultures were made with NCSS software. Data were statistically analyzed with Tukey’s multiple comparison test. Differences of p < 0.05 were considered as significant.

3. Results and Discussion

*Gnaphalium oxyphyllum* is a medicinal plant which is used by Mexican people as an alternative treatment in the cancer disease [17] and there are no scientific studies that support the empiric medical use in the treatment of cancer nor its effect on healthy cells. The cytotoxicity assays are used to know if a plant contains compounds that affect cancer cells survival. For the analysis of cytotoxic activity, cells were treated for 48 h with the decoction from *G. oxyphyllum var. natalie* as well as with some fractions obtained from the CH₂Cl₂: MeOH and MeOH extracts in a concentration range of (100-1.6 μg/mL). Half-maximal inhibitory concentrations (ED₅₀ values) are summarized in Table 1.

The decoction was active to the three cancer cell lines employed (Table 1); the best cytotoxic activity was observed on T47D (breast cancer line cells) cultures. The decoction (ED₅₀ = 4.09 μg/mL) was more active than the precipitate (ED₅₀ = 4.64 μg/mL), the aqueous phase (ED₅₀ = 14.7 μg/mL) and the organic phase ED₅₀ = 10.15 μg/mL of the CH₂Cl₂: MeOH (2:1) extract. Its effect was less than that observed with colchicine (ED₅₀ = 3.06 μg/mL), pure compound with antitumor activity [21], that was used as cytotoxic reference.

On the other hand, the decoction (ED₅₀ = 8.75 μg/mL), the precipitate (ED₅₀ = 11.45 μg/mL), the aqueous phase of the CH₂Cl₂: MeOH extract (ED₅₀ = 14.41 μg/mL), as well the organic phase of the methanol extract (ED₅₀ = 11.63 μg/mL), were cytotoxic

<table>
<thead>
<tr>
<th>Decoction/fraction/compound</th>
<th>T47D (μg/mL)</th>
<th>HeLa (μg/mL)</th>
<th>22Rv1 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decoction</td>
<td>4.09</td>
<td>8.75</td>
<td>13.65</td>
</tr>
<tr>
<td>Precipitate from CH₂Cl₂:MeOH extract</td>
<td>4.64</td>
<td>11.48</td>
<td>NA</td>
</tr>
<tr>
<td>Aqueous phase from CH₂Cl₂:MeOH extract</td>
<td>14.7</td>
<td>92.49</td>
<td>63.12</td>
</tr>
<tr>
<td>Organic phase from CH₂Cl₂:MeOH extract</td>
<td>10.15</td>
<td>15.41</td>
<td>NA</td>
</tr>
<tr>
<td>aqueous phase from MeOH extract</td>
<td>NA</td>
<td>53.91</td>
<td>NA</td>
</tr>
<tr>
<td>Organic phase from MeOH extract</td>
<td>NA</td>
<td>11.63</td>
<td>58.77</td>
</tr>
<tr>
<td>Colchicine</td>
<td>3.93</td>
<td>0.119</td>
<td>0.906</td>
</tr>
</tbody>
</table>

NA: Not assayed. ED₅₀ values ≤ 20 μg/mL are considering as cytotoxic for no pure compounds, meanwhile for pure compounds it value is ≤ 4 μg/mL.
The Effect of *Gnaphalium oxyphyllum var. natalie* on Cancer and Blood Cells

Fig. 1  Blood cell counts, leukocytes, erythrocytes and platelets, in human peripheral lymphocyte cultures did not significantly changed after being exposed for 48 h to different treatments with the *G. oxyphyllum var. natalie* decoction (Average ± SE; Tukey-Kramer, p > 0.05; n = 12).

![Graphs showing blood cell counts](image)

on HeLa culture cells. All of them were less cytotoxic than the positive control (ED$_{50}$ colchicine = 0.119 µg/mL).

Only the decoction was cytotoxic (ED$_{50}$ =13.65 µg/mL) to 22Rv1 line and this effect was less than the positive control (ED$_{50}$ colchicine = 0.906 µg/mL).

Mexican people consume medicinal plants as decoctions and, as our data show the one of *G. oxyphyllum var. natalie* had the highest cytotoxic activity, we decided to investigate its effects on the whole healthy human blood cultures, exposing them during 48 h to the decoction in a concentration range of (2-500 µg/mL). Its effects on leukocyte, erythrocyte and platelet counts were not statistically significant (Fig. 1). Therefore, it did not affect normal blood cells. Only T-lymphocytes proliferated in the cultures due to
The Effect of *Gnaphalium oxyphyllum* var. *natalie* on Cancer and Blood Cells

The mitogenic effects of pythohemaglutinin but all other cells, erythrocytes, platelets and leukocytes (other than T-lymphocytes) remained alive for the 72 h culturing time. This experimental strategy resulted very convenient to this part of the study since it allowed us to test the cytotoxicity of the decoction simultaneously on different cell types. The mitotic index of T-lymphocytes was also evaluated in those cultures. The differences between the control and experimental groups were not significant (Fig. 2) suggesting that none of the concentrations interfered with the normal cell cycle progression. Dividing cells in the experimental groups completed their regular life cycle without progress arrest or delays with respect to the control cultures. Among the experimental groups, there was one significant difference between the cultures exposed to 50 and 500 µg/mL. This result indicates that the minimum toxic concentration of the aqueous extract from *G. oxyphyllum* might be found after 500 µg/mL. Therefore, it will be convenient to perform more cytotoxic tests to exactly determine the point at which the extract becomes toxic. The genotoxicity of the decoction should also be assessed since it has also been determined that the MeOH-CH$_2$Cl$_2$ (1:1) extract of *G. oxyphyllum* at a concentration of 1000 µg per plate significantly increased the number of revertants (His$^-$→His$^+$) of *Salmonella typhimurium* strain TA98 [22]. The observed variability registered in the response among donors is due to the natural biological differences and it has been reported too by other authors [20, 23].

Thus, T-lymphocytes survived and retained their ability to proliferate in the presence of the different concentrations (2-500 µg/mL) of the *G. oxyphyllum* var. *natalie* decoction.

Cancer is the third leading cause of death in Mexico [24]. Since all agents currently being used in cancer therapy are known to be toxic and produce severe damage to normal cells [25], chemotherapy via no toxic medicinal plants could be an alternative to treat cancer.

Toxicity and mutagenic studies performed with inflorescences of *Gnaphalium* spp. showed that the MeOH: CH$_2$Cl$_2$ (1:1) extract did not cause acute toxicity in mice nor in *Artemia salina* (LC$_{50}$ > 1000 µg/mL). However, the Ames test revealed that this extract induced mutation on *Salmonella typhimurium* strain TA98. It is important to point out that this strain is useful for detecting various frameshift mutagens such

![Fig. 2](image)

**Fig. 2** The decoction did not affect the mitotic index in human lymphocyte cultures (Average ± SE; Tukey-Kramer, p > 0.05). Those cultures exposed to 50 and 500 µg/mL significantly differed from each other (Tukey-Kramer, p > 0.026).
as aflatoxins, dimethylbenzantracene, daunomycin and flavonoids [22].

High concentration of the diterpene ent-kaur-16-en-19-oic acid, luteolin and 3-metoxyquercetin [7] have been isolated from *Gnaphalium oxyphyllum* var. *natalie*. This diterpene has already been isolated from some other *Gnaphalium* species [8].

Other compounds that have been isolated from *G. oxyphyllum* are: acetylenic compounds, carotenoid, zoapatlin, 13-epi-cyclosclareol, 5-hydroxy-3,7-dimethoxyquercetin, ent-3-hydroxykaur-16-en-19-oic acid, β-sitosterol, stigmasterol. The flavonoid Kaemperol has also been isolated from *G. oxyphyllum* [26]. Rao et al [25] found that this flavonoid was cytotoxic on Jurkat cells (human leukemia T cells) with ED50 of 48.2 µM and it had not cytotoxic effects at 200 µM concentration on normal peripheral blood mononuclear cells.

*G. gaudichaudianum* DC. is employed in Andean traditional medicine as an expectorant and emmenagogue. One toxicity assay was performed on larvae of the crustacean *Artemia salina* (brine shrimp). The hexane (LC50 = 31 µg/mL), ethyl ether (LC50 = 19 µg/mL) and two diterpenes isolated from methanol extract called as ent-Primara-8(14),15-dien-19-oic acid (LC50 = 27 µg/mL), ent-Prim-15-ene-8α-19-diol (LC50 = 32 µg/mL) were toxic [27]. Velutin (flavone) has also been isolated from this plant.

*G. elegans* Kunth is commonly used to alleviate inflammation of the prostate gland and it is claimed to possess anticancer activity. From its flowers 5,7-dihydroxy-3,6,8-trimethoxyflavone has been isolated but there are not *in vivo* nor *in vitro* studies related to its cancer cell activity [28].

The diterpenes(-)16-kauren-19-oic acid, 11β-acetox-16-kauren-19-oic acid, 13-epi-sclareol, 13-epi-cyclosclareol, sitosterol and stigmasterol have been isolated from *G. graveolens* M. Bieb., a plant used against cancer. However there is nothing reported about its biological activity [8].

*G. americanum* is a botanical synonym of *G. purpureum* L. [2] and with the last botanical name there is a scientific report that shows that the ethanolic extract is not cytotoxic on HCT-116 cells (human colorectal cancer) [29].

There are not previous chemical or biological studies on *G. oxyphyllum* var. *natalie* and, as Arun [30] pointed out, the evaluation of the toxicity of the components of the plants used for medicinal purposes is an important aspect to support their safe use. It would be desirable that toxicity tests could be carried out first *in vitro* in order to avoid unnecessary suffering to the experimental animals.

4. Conclusion

Mexican healers use the decoction of the young steam-leaf of *G. oxyphyllum* var. *natalie* in the treatment of cervical cancer. The experimental data show that this decoction has cytotoxic effect on three human cancer cell lines, one of them of cervical cancer (HeLa). On the other hand, this decoction did not show a negative effect on healthy blood cells in culture.

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The Effect of Gnaphalium oxyphylum var. natalie on Cancer and Blood Cells

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