



Cytotoxic Effects of Flaxseed (*Linum usitatissimum* L.) Aqueous Extract on Different Tumor Cell Lines: An *in vitro* Study

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ABSTRACT:

Mortality rates from cancer are rising continuously worldwide, different methods are used to treat cancer such as chemotherapy, however, chemotherapy is cost ineffective and lacks selective toxicity, since it damages cancerous and healthy normal cells as well, therefore, seeking for natural alternatives is an insisted demand in clinical sitting. Medicinal plants and its derivatives are processed for the direct use as herbal or traditional medicine and attempted for clinical and experimental purposes. Flaxseed (*Linum usitatissimum* L.) is an oilseed utilized in industrial practice as well as natural health products. One of the major traditional interests in flaxseed was its important use in the prevention and treatment of many diseases, including cancer. The current study aimed to investigate the *in vitro* cytotoxic effect of flaxseed aqueous extract on tumor cell lines, and normal cell lines.

Two tumor cell lines (AMN3 and RD) and a normal cell line (L20B), at three time periods (24, 48, and 72 hrs of exposure) with different concentrations (0, 78.125, 156.25, 312.5 and 625 µg/ml), were assessed.

Significant decrease in AMN3 cells viability was detected at the exposure period of 48 hrs in concentrations (156.25 (P= 0.002), 312.5 (P= 0.002) and 625 (P= 0.0004) µg/ml) except 78.125 µg/ml ((P= 0.360), of aqueous flaxseed extract were observed, significant decrease in AMN3 proliferation also observed at 72hrs of exposure in all concentrations of the extract (156.25 (P= 0.000), 312.5 (P= 0.002) and 625 (P= 0.005) µg/ml) except 78.125 µg/ml ((P= 0.328), which shows non-significant differences.

Concerning RD tumor cell line, all applied concentration (0, 78.125, 156.25, 312.5, 625 µg/ml) revealed significant impact on the tumor cell lines at the three applied exposure period, without significant impact on the normal cell line (L20B).

Flaxseed has a concentration, and exposure period dependent impact on the AMN-3 and RD tumor cell lines, *in vitro*. Further studies are needed to detect this anti-proliferative effect and the pathway of flax seed extract in cancer treatment, both *in vitro* and *in vivo*.

Keywords: AMN-3 cell line; Aqueous Extract; Cytotoxic effect; Flaxseed; L20B; Rhabdomyosarcoma (RD) cell line.



1 INTRODUCTION

The second leading cause of death in the world is cancer. Cancer is defined as uncontrolled growth of body cells that have had profound damage in their DNA expressions. If this abnormal cell growth is not managed accordingly to the cause, it can lead to various complications and death. The atypical cells are named as cancerous cells (malignant cells, or tumor cells). Different types of cancers are further classified by the organ of origin (for example, breast cancer, lung cancer, prostate cancer, and colorectal cancer). Other kinds of cancers depend on the type of genes associated with it like sarcomas, carcinomas, leukemia, and lymphomas. Carcinogenesis consists of three distinguished stages, (initiation, promotion, and progression) [1] [2].

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Mortality rates from cancer are rising continuously worldwide in 2022, there was roughly 20 million new cases of cancer and 9.7 million deaths due to the disease. The number of people who were alive in a time span of 5 years following a cancer diagnosis was roughly 53.5 million. Almost 1 in every 5 people develop cancer in their life time, approximately 1 in 9 men and 1 in 12 women die from the mentioned disease. Nowadays, numerous methods are used to treat cancer such as chemotherapy, however chemotherapy is a non-selective method, because a high number of healthy cells will be targeted and damaged concurrently with cancer cells. The most important goal in different types of cancer treatment is destroying tumor cells in the presence of normal cells, without damaging them [3].

Due to the rising incidence of the disease and the high cost of treatment, there has been a growing interest in developing new strategies for preventing and treating cancer. One of these is the use of natural substances as chemopreventive or anticancer agents [2]

A large number of cancer patients seek treatments with complementary and/or alternative medicine, particularly in cases that have developed drug resistance towards chemotherapies [2].

In the case of this anticancer medication which are prepared from natural resources such as plants, by testing the cytotoxic compounds of different parts of plants and screening raw extracts of plants is necessary. Medicinal plants are important for treatment of cancer because they contain multiple chemical compounds and are a new side for discovering new active materials against cancer [3]. Medicinal plants and its derivatives are processed for the direct use as herbal or traditional medicine as well as being prepared for experimental purposes [4].

Flax (*Linum usitatissimum* L.), also known as linseed, belongs to the *Linaceae* family which originates from Europe, Asia, Flaxseed is an oilseed utilized in industrial practice as well as natural health products. Flaxseeds have many different functions whether utilized industrially or in food. Flax is used in the production of dietary products in the Mediterranean region. It is considered a functional food that has nutrients with specific contains, omega-3 fatty acids that promote the reduction of cholesterol levels, thus preventing cardiovascular diseases, α -linolenic acid (LA), lignan which are phytoestrogens that relieve the symptoms of menopause and can balance the effects of estrogen in the body by connecting to their receptors, as they have a very similar chemical structure as to an estrogen molecule. The predominant lignan in flaxseeds is secoisolariciresinol diglucoside (SDG), making up around 95% of the seed's lignan content. The remaining 5% consist of lariciresinol, pinoresinol, and matairesinol. After SDG lignan ingestion, bacteria in the colon act by converting the lignan into mammalian lignans, enterolactone, and enterodiol. These are structurally similar to estrogen, and have antioxidant activity and a weak estrogenic action. It also works as an antiestrogenic because its structure is very similar to the main form of estrogen, which allows its binding to the cell's receptors, thus inhibiting the growth of cancer cells. Flaxseeds are rich in fiber and are suggested for situations of constipation, as they help to improve the intestinal function. Additionally, they are still a good source of magnesium, phosphorus, manganese, vitamin B1, selenium, and zinc [2, 5].

One of the major traditional interests in flaxseed was its important use in the prevention and treatment of many diseases, including respiratory tract, skin, gastrointestinal tract infections, hepatic disorders and cancer [6].

Most of the studies regarding flaxseed focus on its extracts, which contain α -linolenic acid or lignan. While the Other flaxseed compounds have gained less attention and their activity is not described as thoroughly. The advantages of using the whole flaxseed fractions such as oil, mucilage and protein indicates the consideration of the entire portfolio of bio actives present is required to associate biological activity with specific compounds [5] [7].

Lignans are polyphenols that can act like both antioxidants and phytoestrogens. Their content in flaxseed is principally composed of seco-isolariciresinol diglucoside (SDG), pinoresinol, lariciresinol and matairesinol, respectively from highest to lowest concentration, as lignans (through antitumor, anti-oxidant, both estrogenic, anti-estrogenic activity), modulate the signaling mechanism of cell proliferation, inflammation, and oxidative stress and thereby can reduce the risk of cancer or its progression. [2] [6] [8].

Flaxseeds as a whole and its oil components have been observed to exhibit various activities and reactions against different types of cancer; making flaxseed a complementary and/or alternative medicine candidate for human cancer treatment [9].

To further investigate the organic components of flaxseeds, scientists have had it characterized into a water-soluble extract, as well as reporting an abundance of proteins, water soluble carbohydrates, phenolic compounds, essentially SDG, ferulic acid and p-coumaric acid. Flaxseeds have the highest content of lignans of all plant foods, up to 800 times more than other plant foods used for human consumption [10] [11].

Since flaxseed has different anticancer and antioxidant effect it has been taken by people normally as a diet or prepared by cooking or as a hot drink, in the current study we tested the cytotoxicity of flaxseed aqueous extract on two tumor cell lines and a normal cell line in order to detect its cytotoxic effects.

2 MATERIAL AND METHODS

PREPARATION OF AQUEOUS EXTRACTS FROM FLAX SEEDS

The dried flax seeds (50 grams) were ground into powder using an electric grinder with a mesh size of 0.5 mm. The resulting powder was then stored in plastic tubes and stored at -20°C in a deep freezer until use. An aqueous extract was prepared from the ground powder following the method outlined in [12]. Once the crude dried extract was prepared, it was placed in labeled plastic tubes that were tightly sealed and stored at -20°C until further use. For the subsequent experiments, one gram of seed extract was dissolved in 100 ml of phosphate buffer saline (PBS) for each preparation. The suspension was then filtered and sterilized with a $0.2\text{ }\mu\text{m}$ sterile Millipore filter and preserved in a deep freeze at (-20°C) until utilized.

CELL LINES

This study utilized two distinct tumor cell lines: Rhabdomyosarcoma (RD) cell line, which originates from a biopsy of a pelvic rhabdomyosarcoma in a 7-year-old Caucasian female [13], and the Ahmed-Mohammed-Nahi-2003 (AMN-3) cell line, derived from a spontaneous mammary adenocarcinoma in female BALB/c mice. Additionally, a normal cell line, the L20B cell line, was included; this murine line is from mouse L fibroblasts that express the human poliovirus receptor [14].

All cell lines were cultured in RPMI-1640 medium (Rosewell Park Memorial Institute, SIGMA-Aldrich, St. Louis, MO, USA), supplemented with 10 % FBS (Fetal Bovine Serum, Gibco, Dublin, Ireland) and 100 U/mL Penicillin and 100 $\mu\text{g/mL}$ Streptomycin in a 5% CO_2 humidified incubator at 37°C .

CYTOTOXICITY ASSAY

The cytotoxicity method followed the guidelines set forth by Flick and Gifford (1984) [15]. Cells were seeded in a 96-well flat-bottom plate, and once they adhered, serial dilutions of the aqueous extract were added in triplicate for each concentration (625, 312.5, 156.25, 78.125, and 0 $\mu\text{g/mL}$) across three columns, with 200 μL of each extract placed into the respective wells and incubated for 24, 48 and 72 hrs at 37°C , 5-10% CO_2 in a humidified environment. Untreated cells (0 $\mu\text{g/mL}$) served as controls. After incubation, the supernatants were removed, the cells in each well were washed twice with PBS, and 50 μL of 0.01% neutral red dye was added to each well, followed by an additional re-incubation for 2hrs. At the end of this period, excess dye was removed by washing the wells twice with 150 μL PBS, after which 125 μL of extraction dye solution was added [16]. The optical density (OD) for each well was measured with an enzyme-linked Immunosorbent assay (ELISA) reader at a transmission wavelength of 492 nm.

STATISTICAL ANALYSIS

The significance level was determined through a one-way analysis of variance (Anova), followed by multiple Student-Newman-Keuls tests. Results were reported as the mean $\pm$ standard deviations of the mean of optical density at 492 nm. A p-value below 0.05 was deemed statistically significant. All statistical analyses were conducted using SPSS software (version 26).

3 RESULTS

CYTOTOXIC EFFECT OF FLAXSEEDS AQUEOUS EXTRACT ON AMN3 TUMOR CELL LINE

AMN3 tumor cell line which is a murine mammary adenocarcinoma cell line derived from a spontaneous mammary adenocarcinoma of female BALB/c mice, After 24 hrs of exposure there weren't any significant differences in cell viability rate in all concentrations of flaxseed, while there was a significant decrease in cell viability at the exposure time 48hrs in all three other concentration except 78.125 $\mu\text{g/mL}$, while when the cells exposed to the same concentrations but for 72 hrs there was a significant decrease in the viability of cells respectively with concentration, this effect is clear in Table 1, and in Figure 1.

CYTOTOXIC EFFECT OF FLAXSEEDS AQUEOUS EXTRACT ON RD TUMOR CELL LINE

The cytotoxic effect of aqueous extract of flaxseeds on the growth of RD tumor cell line is shown in Table 2, A significant decrease in cell proliferation was detected in all concentrations of the extract at all three time periods of exposure, except exposure to 625($\mu\text{g/mL}$) for 24 hrs which did not show any significant ($P= 0.08815$) differences.

As its clear that the cell viability at 24 hrs and 48 hrs of exposure respectively, was lowest at 10000 $\mu\text{g/mL}$ was (0.1059 ± 0.00298) and (0.1198 ± 0.00024) in which there is a significant decrease in cell concentration and viability when compared with their control. Figure 2 shows the changes in cells such as shape and accumulation of cell west products inside the culture and a large number of them takes rounded shape and as its clear in the figures that in the highest concentrations of the extract have the most effective action on the cells and causes death of almost all of them.

CYTOTOXIC EFFECT OF FLAXSEEDS AQUEOUS EXTRACT ON L20B CELL LINE

Table 3 shows the cytotoxic effects of flaxseed extract on normal cells L20B, non-significant differences were detected in both period of exposure time 24 hrs and 48 hrs for all concentrations (figure 3). At 72hrs of exposure there was a significant increase in cell proliferation and this increase was concentration depending increase.

Table 1. Cytotoxic effect (mean $\pm$ standard error of optical density at 492 nm) of aqueous extract of flaxseeds on the growth of AMN3 tumor cell line.

Concentrations ($\mu\text{g/ml}$)	Exposure period		
	24 hrs	48 hrs	72 hrs
Control	0.062 $\pm$ 0.002	0.227 $\pm$ 0.000	0.226 $\pm$ 0.001
78.125	0.061 $\pm$ 0.011 (P= 0.490)*	0.224 $\pm$ 0.010 (P= 0.360)*	0.221 $\pm$ 0.016 (P= 0.328)*
156.25	0.061 $\pm$ 0.002 (P= 0.331)*	0.115 $\pm$ 0.0769 (P= 0.002)*	0.1121 $\pm$ 0.013 (P= 0.000)*
312.5	0.066 $\pm$ 0.008 (P= 0.210)	0.097 $\pm$ 0.006 (P= 0.0002)*	0.182 $\pm$ 0.006 (P= 0.002)*
625	0.064 $\pm$ 0.005 (P= 0.269)*	0.115 $\pm$ 0.006 (P= 0.0004)*	0.121 $\pm$ 0.019 (P= 0.005)*
	(P= 0.777)** (F: 0.368; F crit: 4.0661)	(P= 0.000)** (F: 113.85; F crit: 4.0661)	(P= 0.000)** (F: 42.634; F crit: 4.0661)

*Test versus control

**Anova single factor among different concentrations in a given exposure time.

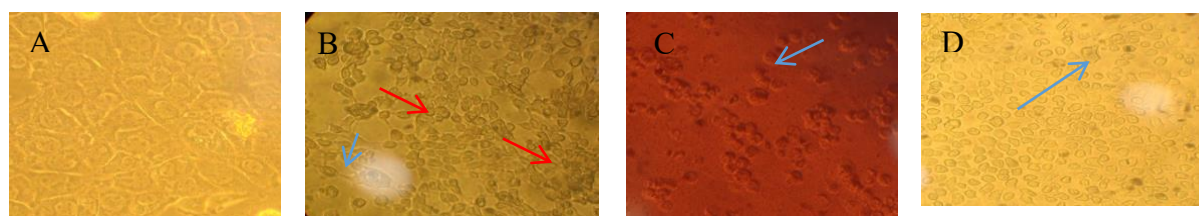


Figure 1. The cytotoxic effect of flaxseed aqueous extract on Amn3 tumor cell lines at different times and different concentrations. A- control. B- exposure time 48hrs (625 $\mu\text{g/ml}$). C- exposure time 72 hrs (625 $\mu\text{g/ml}$). D- exposure time 24hrs (625 $\mu\text{g/ml}$). Blue arrow- rounded cells. Red arrow- cellular accumulation.

Table 2. Cytotoxic effect (mean $\pm$ standard error of optical density at 492 nm) of aqueous extract of flaxseeds on the growth of RD tumor cell line.

Concentrations ($\mu\text{g/ml}$)	Exposure period		
	24 hrs	48 hrs	72 hrs
Control	0.3633 $\pm$ 0.0533	0.4430 $\pm$ 0.0060	0.1128 $\pm$ 0.00148
78.125	0.2996 $\pm$ 0.0005 (P= 0.003)*	0.3077 $\pm$ 0.00240 (P= 0.000)*	0.0938 $\pm$ 0.00317 (P= 0.007)*
156.25	0.3027 $\pm$ 0.00600 (P= 0.000)*	0.2887 $\pm$ 0.00186 (P= 0.000)*	0.0881 $\pm$ 0.00127 (P= 0.000)*
312.5	0.3086 $\pm$ 0.00391 (P= 0.000)*	0.2641 $\pm$ 0.00012 (P= 0.000)*	0.0961 $\pm$ 0.00124 (P= 0.000)*
625	0.3524 $\pm$ 0.00020 P= 0.08815	0.2741 $\pm$ 0.00035 P= 0.00062	0.0976 $\pm$ 0.00038 P= 0.003275
	P= 0.000 (F: 47.269; F crit: 4.0661)**	P= 0.000 (F: 153.499; F crit: 4.0661)**	P= 0.02754 (F: 5.2131; F crit: 4.0661)**

*Test versus control

**Anova single factor among different concentrations in a given exposure time.

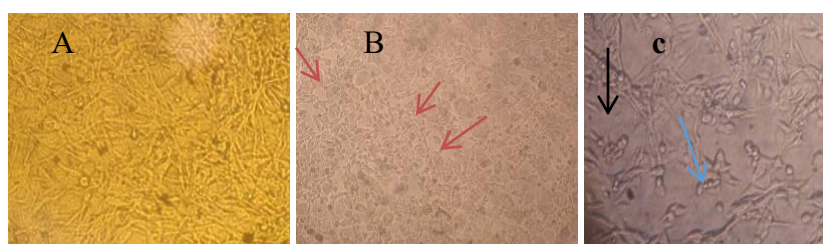
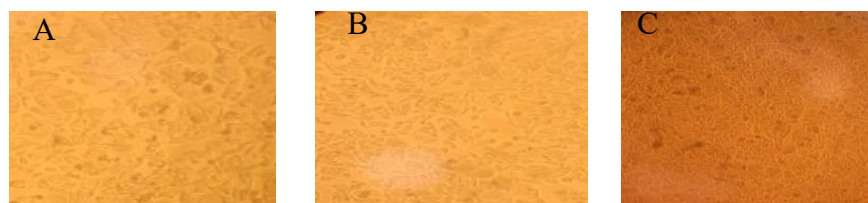
**Figure 2.** The cytotoxic effect of flaxseed aqueous extract ($\mu\text{g/ml}$) on RD tumor cell lines at different times and different concentrations. A-control. B- exposure time 48 hrs (312.5 $\mu\text{g/ml}$). C- exposure time 72 hrs (312.5 $\mu\text{g/ml}$). Red arrow- multi-nucleated cells. Blue arrow- rounded cells. Black arrow- cellular accumulation

Table 3. Cytotoxic effect (mean $\pm$ standard error of optical density at 492 nm) of aqueous extract of flaxseeds on the growth of L20B cell line.

Concentrations ($\mu\text{g/ml}$)	Exposure period		
	24 hrs	48 hrs	72 hrs
Control	0.1009 $\pm$ 0.00003	0.1037 $\pm$ 0.0008	0.574 $\pm$ 0.0066
78.125	0.0743 $\pm$ 0.00296 (P= 0.006)*	0.0900 $\pm$ 0.001 (P= 0.000)*	0.6376 $\pm$ 0.0024 (P= 0.000)*
156.25	0.0091 $\pm$ 0.0028 (P= 0.0139)*	0.0886 $\pm$ 0.0055 (P= 0.0198)*	0.6256 $\pm$ 0.0090 (P= 0.000)*
312.5	0.0769 $\pm$ 0.00329 (P= 0.004)*	0.0913 $\pm$ 0.0023 (P= 0.0031)*	0.6294 $\pm$ 0.0092 (P= 0.000)*
625	0.0716 $\pm$ 0.002 (P= 0.000)*	0.0936 $\pm$ 0.0059 (P= 0.0486)*	0.6120 $\pm$ 0.0011 (P= 0.0043)*
	(P= 0.000)** (F: 17.284; F crit: 4.0661)**	(P= 0.553)** (F: 0.747; F crit: 4.0661)**	(P= 0.009)** (F: 7.847; F crit: 4.0661)**

*Test versus control

**Anova single factor among different concentrations in a given exposure time

**Figure 3. The cytotoxic effect of flaxseed aqueous extract on L20B cell lines at different times and different concentrations. a- control. b- exposure time 48 hrs (625 $\mu\text{g/ml}$). c-exposure time 72 hrs (312.5 $\mu\text{g/ml}$).**

4 DISCUSSION

The results showed a significant decrease in cell viability in both type of tumor cell lines AMN3 & RD when treated with different concentrations of flaxseed extract and incubated for three time period but this effect was clear in both 48 & 72 hrs of exposure and high concentrations was most effected one, the results are clear in tables (1&2), figures (1&2).

This action of flaxseed extract may be due to the presence of lignans which are a group of secondary metabolites found in different plants exhibit different biological activities; that include cytotoxicity and anti-cancer effects.it was shown that flaxseeds contain a high percentage of these lignans. [8].

Enterolactone a type of lignans that is found in flaxseeds have growth-inhibitory effects that were found in three lung cancer cell lines, by down-regulating G1-phase cyclins and CDKs, and up-regulating p21, which leads to G1-phase cell cycle arrest [9].

The anti-proliferative effect might be also due to the presence of omega-3 and α -linolenic acid (ALA), which are also constituents of flaxseed. Flaxseed is to be considered as the best plant origin of the essential omega-3 fatty acid. Studies have implied that the omega-3 fatty acid might have anticancer properties while the omega-6 fatty acid can promote the development of cancer [8].

The anticancer properties of flaxseed oil were also shown by [17], which examined its direct effects on the growth of cancer cells *in vitro*; treating various cancer cell lines with flaxseed oil reduces their growth in a dose-dependent manner, and it was observed that ALA, DHA, EPA, and the lignans (ENL and END) inhibited cell growth. Another component of flaxseed that demonstrates anticancer effects is Linoorbitides, which are naturally occurring, hydrophobic, thermostable cyclopeptides that exhibit antioxidant properties in flaxseed oil and also hold potential for antitumor activity. Zou et al. (2019) [18] investigated the anti-proliferative effect of two flaxseed orbitides using gastric SGC-7901 cells. They discovered that the two linoorbitides could potentially induce cell cycle arrest in the G1 phase of SGC-7901 cells in a dose-dependent manner, which was associated with the down regulation of CDK2, CDK4, cyclin D3, and cyclin.

E. Okinyo-Owiti et al. (2015) assessed the cytotoxic effects of linoorbitides (compounds from flaxseed) known for their anticancer and antioxidant properties against human breast cancer (SkBr3 and MCF-7) cell lines, as well as the melanoma (A375) cell line. The study showed that the level of cytotoxicity relied on the concentration used.

Di et al. (2018) [19] investigated the cytotoxic effects of combining flaxseed lignans with traditional chemotherapeutic drugs on metastatic breast cancer cell lines and found that flaxseed lignans notably increased the effectiveness of these drugs against breast cancer cells.

Musavi et al. (2022) [20] found that the flaxseed aqueous extract has a good cytotoxic effect on colon tumor cell lines and can decrease its viability, while no significant decrease in cell viability was observed in normal cells. The cells were treated for two days with different concentrations of the extract. They found that seed aqueous extract has anti-proliferative and pro-apoptotic properties and can halt cancer growth and promote apoptosis while not harming normal cells in the process.

Flaxseed has the potential to enhance cell apoptosis and suppress tumor development; thus, it may be useful in preventing cancer's onset and slowing its progression. Additionally, it can boost the effectiveness of chemotherapy drugs used by cancer patients[5].

According to the effect of flaxseed extract on normal cell line a significant increase was detected in all concentrations with time in which non-significant differences was detected in 24 hrs of exposure but a significant increase detected in both 48 & 72 hrs. of exposure, this time depending response was detected by Shaban *et al.*, [21] they found that crude flaxseed oil have high wound healing potential on human oral fibroblast cells, and the cells re-grew as fast as 18-hour post-treatment with compared to cells with no treatment. The full coverage of the wound was achieved within 72-hour post-treatment in most areas of the wound. Terpenoids, oleic and linolic acid have been reported to promote the wound healing process and induce protective effects in cells [22].

Flaxseed is a therapeutically important plant that contains compounds with activities that are beneficial to human health.

CONCLUSION

Flaxseed aqueous extract shows a cytotoxic effect on growth of both types of tumor cell lines (AMN3& RD) and the effect was time and concentration depending, but the same concentrations of the extract causes increase in proliferation of L20B cell line, it is clear that the extract decrease proliferation of tumor cell lines with no such effect on normal cells, further studies are recommended to detect the anti-proliferative effect and the pathway of flax seed extract in cancer treatment, both *in vitro* and *in vivo*.

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CONFLICTS OF INTEREST

The author declares no conflict of interest.

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