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Assessment of endocrine disruption potential of essential oils of culinary herbs and spices involving glucocorticoid, androgen and vitamin D receptors

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ABSTRACT

Essential oils (EOs) of culinary herbs and spices are consumed on common bases. They are multicomponent mixtures of compounds with already demonstrated biological activities. Taking in account regular dietary intake and the chemical composition of EOs, these may be candidates for endocrine disrupting entities. Therefore, we examined the effects of 31 EOs of culinary herbs and spices on the transcriptional activities of glucocorticoid receptor (GR), androgen receptor (AR) and vitamin D receptor (VDR). Using reporter gene assays in stably transfected cell lines, weak anti-androgen and anti-glucocorticoid activity was observed for EO of vanilla and nutmeg, respectively. Moderate augmentation of calcitriol-dependent VDR activity was caused by EOs of ginger, thyme, coriander and lemongrass. Mixed anti-glucocorticoid and VDR-stimulatory activities were displayed by EOs of turmeric, oregano, dill, caraway, verveine and spearmint. Remaining 19 EOs were inactive against all receptors under investigation. Analyses of GR, AR and VDR target genes by the means of RT-PCR confirmed VDR-stimulatory, but not anti-glucocorticoid and anti-androgen effects of EOs. In conclusion, while we observed minor effects of several EOs on transcriptional activities of GR, AR and VDR, the toxicological significance is very low. Hence, 31 EOs of culinary herbs and spices may be considered safe, in terms of endocrine disruption involving receptors GR, AR and VDR.

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INTRODUCTION

Essential oils (EOs), are massively used in cosmetics, medicine, food industry and gastronomy. In the latter applications, EOs of culinary herbs and spices are used to flavor, color and preserve foods and drinks. They are concentrated hydrophobic liquids, containing mainly volatile constituents, such as ethers, esters, alcohols, terpenes, aldehydes, hydrocarbons etc. [1]. Large body of evidence supports various biological activities of EOs, including anti-microbial [2], anti-fungal [3], anti-inflammatory [4], anti-diabetic [5], anti-hypertensive [6] and many others. There is an increasing dietary intake of EOs, which is also documented by a number of available cookery books and recipes using EOs as ingredients. Given the chemical complexity of EOs, their significant consumption in a diet and the spectrum of yet demonstrated biological activities, it is beneficial and essential to assess putative endocrine disruptive potential of EOs of culinary herbs and spices. Endocrine disrupting chemicals (EDC) are defined as those interfering with endocrine (hormonal) systems in human body, thereby, causing developmental defects, birth defects and in general pathologies dependent on hormonal dysregulation including diabetes, obesity, cancers, auto-immune disorders, fatty-liver disease etc [7]. The representatives of EDCs are structurally unrelated chemicals involving phthalates (plasticizers), polychlorinated biphenyls (pesticides), dioxins (industrial by-products), polybrominated diphenyl ethers (flame retardants), organotin derivatives (dyes and paints) and many others. These compounds originate mainly from environmental pollution, but they are also of natural origin contained in foods and drinks (e.g. isoflavones). The molecular mechanism of EDCs action is either inhibition of key enzymes involved in homeostasis of hormones (e.g. 11β-hydroxysteroid dehydrogenase) [8] or interference with nuclear receptors, receptors for steroid hormones and xenoreceptors. The most prominent soluble receptor targets for EDCs are: (i) nuclear receptors: thyroid hormone receptor, retinoic acid receptors (RARs), retinoid X receptors (RXRs), vitamin D receptor

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(VDR), peroxisome proliferator activated receptors (PPARs); (ii) steroid hormones receptors: androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR); (iii) xenoreceptors: aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR).

Effects of EOs of culinary herbs and spices on transcriptional activities of the soluble receptors mentioned above were studied sporadically. Takahashi et al described that seed extracts and EO of dill suppress high-fat diet-induced hyperlipidemia in rats through hepatic PPAR-α activation [9]. Citral, a component of lemongrass oil [10] and carvacrol, a component of thyme oil [11] were found to activate human PPARα and PPARγ. Prevention of chemically-induced hyperthyroidism was described in rats administered with EO of Satureja khuzestanica [12]. Common constituents of several EOs, including citral, geraniol, nerol and eugenol were able to displace estradiol from binding to ER, however, biological significance was disputable since neither estrogenic nor anti-estrogenic activities were demonstrated in estrogen-responsive human cell line Ishikawa Var I and in vivo in ovarectomized mice [13]. However, measured end-points were uterine hypertrophy and acute increase in uterine vascular permeability, which is not truly relevant to estrogen receptor beta activity.

No literary data are available on the effects of EOs of culinary herbs and spices on VDR, AR, GR, RXRs, RARs and PXR.

We have recently described the effects of EOs of culinary herbs and spices on AhR-CYP1A1 signaling pathway. We identified EOs having AhR-full agonist activity (cumin, jasmine, vanilla, bay leaf), AhR-partial agonist activity (cloves, dill, thyme, nutmeg, oregano) and AhR-antagonist activity (tarragon, caraway, turmeric, lovage, fennel, spearmint, star anise, anise). We also studied major constituents of AhR-active EOs, and we identified AhR partial agonists (carvacrol, ligustilide, eugenol, eugenyl acetate, thymol, *ar*-turmerone) and antagonists (*trans*-anethole, butylidine phtalide, R/S-carvones, *p*-cymene), which account for

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AhR-mediated activities of EOs of fennel, anise, star anise, caraway, spearmint, tarragon, cloves, dill, turmeric, lovage, thyme and oregano [14].

In the current paper, we have examined the effects of 31 EOs of culinary herbs and spices on common transcriptional targets for endocrine disruptors. To make a study straightforward and swift, we selected AR, GR and VDR as representatives of sex hormone-activated steroid receptor, corticoid hormone-activated steroid receptor and nuclear receptor, respectively. By the means of reporter gene assays in stably transfected transgenic cell lines AZ-GR [15], AIZ-AR [16], IZ-VDRE [17] and RT-PCR analyses of target genes. Cell lines AZ-GR, AIZ-AR and IZ-VDRE were developed as highly sensitive and selective tools for the assessment of transcriptional activities of human receptors GR, AR and VDR [15-17], as already demonstrated on the cases of anthocyanidines [18], mixed-ligand Cu(II) complexes [19] or Au(I) complexes [20]. We concluded here that despite minor effects of several EOs on transcriptional activities of GR, AR and VDR, the toxicological significance of these effects is very low. Hence, 31 EOs of culinary herbs and spices may be considered safe, in terms of endocrine disruption involving receptors GR, AR and VDR.

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MATERIALS AND METHODS

Chemicals

EOs of dill (*Anethum graveolens*; fruit), tarragon (*Artemisia dranunculus*; flowering top), caraway (*Carum carvi*; seed), cinnamon (*Cinnamomum zeylanicum/verum*; bark), coriander (*Coriandrum sativum*; leaf), cumin (*Cuminum cyminum*; fruit), turmeric (*Curcuma longa*; root), lemongrass (*Cymbopogon citratus*; flower), cardamom (*Elletaria cardamomum*; fruit), cloves (*Eugenia caryophyllus*; bud), fennel (*Foeniculum vulgare*; flowering top); star anise (*Illicium verum*; fruit), jasmine (*Jasminum officinalis*; blossom), juniper (*Juniperus communis ssp communis*; twig and berries), bay leaf (*Laurus nobilis*; leaf), lovage (*Levisticum officinale*; root); verveine (*Lippia citriodora*; leaf), cornmint (*Mentha arvensis*; flower), spearmint (*Mentha spicata*; flower), peppermint (*Mentha x piperita*; flower), nutmeg (*Myristica fragrans*; fruit), basil (*Ocimum basilicum*; flowering top), oregano (*Origanum compactum*; flowering top), marjoram (*Origanum majorana*; flowering top), black pepper (*Piper nigrum*; fruit), rosemary (*Rosmarinus officinalis* ct cinéole; flowering top), sage (*Salvia officinalis*; flowering top), thyme (*Thymus vulgaris* ct thymol; flowering top), vanilla (*Vanilla fragrans Auct*; oleoresine), and ginger (*Zingiber officinale*; rhizome) were purchased from Pranarôm (Ghislenghien, Belgium). The quality of EOs was checked for the absence of organochlorine (GC/MS/XSD) and organophosphate (GC/MS/FPD) pesticides (by Pranarôm). The quality of EOs was checked for the absence of organochlorine (GC/MS/XSD) and organophosphate (GC/MS/FPD) pesticides (by Pranarôm). Detailed composition of EOs (listing constituents > 1%) was published previously [14]. EO of anise (*Pimpinella anisum*), dimethylsulfoxide (DMSO), dexamethasone (DEX), 5α-dihydrotestosterone (DHT) and charcoal-stripped fetal bovine serum were from Sigma Aldrich (Prague, Czech Republic). Hygromycin B and 1α,25-dihydroxyvitamin D3 (calcitriol) were from SantaCruz Biotechnology (Santa Cruz, CA,

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USA). Reporter Lysis Buffer was from Promega (Hercules, CA, USA). All other chemicals were of the highest quality commercially available.

Cell lines

Human Caucasian colon adenocarcinoma cell line LS180 (ECACC No. 87021202), Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430), Human cervix epitheloid carcinoma cells HeLa (ECACC No. 93021013), and Human prostate carcinoma epithelial cells 22Rv1 (ECACC No. 105092802) were purchased from the European Collection of Cell Cultures (ECACC). Transgenic reporter gene cell lines AZ-GR, AIZ-AR and IZ-VDRE, allowing assessment of transcriptional activities of glucocorticoid receptor (GR), androgen receptor (AR) and vitamin D receptor (VDR), respectively, were described elsewhere [15-17]. LS180, HepG2, HeLa, AZ-GR, and IZ-VDRE cell lines were cultivated in Dulbecco's modified Eagle's medium DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1% non-essential amino acids and 1 mM sodium pyruvate. AIZ-AR and 22Rv1 cells were cultured in Roswell Park Memorial Institute medium RPMI-1640 supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

Cell viability assay

Cells were incubated with EOs 0.01 µg/mL - 250 µg/mL), vehicle (UT; 0.1% v/v ethanol) and Triton X-100 (1%, v/v), using 96-wells culture plates. MTT assay was performed applying incubation time of 2 h and absorbance was measured at 570 nm on Infinite M200 (Schoeller Instruments, Prague, Czech Republic). The data were expressed as the percentage of cell viability, where 100% and 0% represent the treatments with negative control (EtOH) and positive control (Triton X-100), respectively.

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Reporter gene assay

The stably transfected gene reporter cell lines AZ-GR, AIZ-AR and IZ-VDRE were used for the valuation of transcriptional activity of GR, AR and VDR, respectively. AZ-GR, AIZ-AR and IZ-VDRE cells were seeded in 96-well plates at a density of 2×10^4 cells/well, 5×10^4 cells/well and 2.5×10^4 cells/well, respectively. Following 24 h of stabilization, cells were incubated for 24 h with essential oils and vehicle (EtOH; 0.1% v/v) in the presence (antagonist mode) or in the absence (agonist mode) of model ligands for GR (DEX), AR (DHT) and VDR (calcitriol). Thereafter, the cells were lysed and luciferase activity was measured on Tecan Infinite M200 Pro plate reader (Schoeller Instruments, Czech Republic).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The total RNA was isolated by TRI Reagent® (Sigma-Aldrich, USA) and cDNA was synthesized using 1 μg of mRNA according to the common protocol using M-MuLV Reverse Transcriptase (New England Biolabs, USA) at 42°C for 60 min in the presence of random hexamers (New England Biolabs, USA). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using 2 μL of a product at LightCycler ® 480 Probes Master on a Light Cycler ® 480 II apparatus (Roche Diagnostic Corporation). The levels of *CYP24A1*, *TAT*, *PSA* (*KLK3*), and *GAPDH* mRNAs were determined using Universal Probes Library (UPL; Roche Diagnostic Corporation) probes and primers as described in Table 1. The following protocol was used: an activation step at 95 °C for 10 min was followed by 45 cycles of PCR (denaturation at 95 °C for 10 s; annealing with elongation at 60 °C for 30 s). Measurements were performed in triplicates. The data were processed by the delta-delta method and they were normalized *per GAPDH* as a housekeeping gene.

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Statistics

Student t-test, one-way analysis of variance (ANOVA), and Dunnett test, and calculations of EC_{50} and IC_{50} values were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA).

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RESULTS

Effects of EOs on transcriptional activity of glucocorticoid receptor

Transcriptional activity of GR was assessed in transgenic, stably transfected reporter gene cell line AZ-GR, derived from Human cervical carcinoma cells HeLa [15]. Studied EOs were tested in concentrations relevant to a real exposure from food intake. We incubated cells with EOs for 24 h, in concentrations range from 0.01 μg/mL to 250 μg/mL. Prior to the reporter gene assays, we assayed the cytotoxicity of tested EOs in HeLa cells, by the means of MTT test. The majority of tested EOs were not cytotoxic even in the highest applied concentration (250 μg/mL). Dose-dependent decrease in cell viability was observed for EOs of cinnamon, cloves, coriander, lemongrass, lovage, oregano, spearmint, thyme, turmeric and verveine (Figure 1A).Therefore, reporter gene assays were carried out using EOs in concentrations that caused decline in the viability of HeLa not greater than 30%. Incubations were performed in the absence or in the presence of dexamethasone (DEX), referred to as an agonist and an antagonist mode, respectively. Induction of luciferase activity in four consecutive cell passages of AZ-GR cells by DEX (100 nM), which is a prototypical and potent agonist of GR, ranged from 49-fold to 127-fold, as compared to vehicle-treated cells. None of the EOs tested did activate GR in this assay (Figure 1B). The concentration of DEX, used in antagonist mode, was selected based on dose-response analysis, when the induction of luciferase activity by 100 nM DEX was in ascending part of the curve, close to EC_{50} value (30.3 ± 4.8 nM; n = 5; see inserted plots in Figure 1C). Significant anti-glucocorticoid activity, unrelated to cytotoxic effects of EOs, was observed for EOs of caraway, dill, nutmeg, oregano, spearmint, turmeric and verveine (Figure 1C).

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Effects of EOs on transcriptional activity of androgen receptor

Effects of EOs on transcriptional activity of AR were assessed in stably transfected reporter gene cell line AIZ-AR, derived from Human prostate carcinoma epithelial cell line 22Rv1 [16]. Cells were incubated with EOs for 24 h, in concentrations ranging from 0.01 μg/mL to 250 μg/mL. We tested the cytotoxicity of EOs in 22Rv1 cells using MTT assay. Similarly as in HeLa cells, some of the tested EOs were cytotoxic also in 22Rv1, and dose-dependent decrease in cell viability was observed for EOs of cinnamon, cloves, coriander, lemongrass, lovage, oregano, spearmint, thyme, turmeric and verveine (Figure 2A). Reporter gene assays in AIZ-AR cells were carried out using EOs in concentrations that caused decline in the viability of 22Rv1 not greater than 30%. Incubations were carried out in the absence and in the presence of 5α-dihydrotestosterone (DHT; 100 nM), which is a prototypical and potent agonist of AR. The concentration of DHT, used in antagonist mode, was selected based on dose-response analysis, when the induction of luciferase activity by 100 nM DHT was in ascending part of the curve, adjacent to the saturation part (0.58 ± 0.02 nM; n = 5; see inserted plots in Figure 2C). The induction of luciferase activity in four consecutive cell passages of AIZ-AR cells by DHT ranged from 14-fold to 17-fold, as compared to DMSO-treated cells. None of the EOs tested did activate AR in this assay (Figure 2B). Significant, but weak, anti-androgen activity, unrelated to intrinsic cytotoxicity, was observed for EO of vanilla (Figure 2C).

Effects of EOs on transcriptional activity of vitamin D receptor

Effects of EOs on transcriptional activity of VDR were examined in transgenic reporter gene cells IZ-VDRE, derived from Human intestinal carcinoma cells LS180 [17]. Cells were incubated with EOs for 24 h, in concentrations ranging from 0.01 μg/mL to 250 μg/mL. Similarly as in HeLa and 22Rv1 cells, the cytotoxicity of EOs of cinnamon, cloves, coriander,

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lemongrass, lovage, oregano, thyme, turmeric and verveine was also observed in LS180 (Figure 3A). A cut-off for performing reporter gene assays in IZ-VDRE cells was decline in the viability of LS180 cells not greater than 30%. Incubations were carried out in the absence and in the presence of calcitriol (50 nM), which is a prototypical and potent agonist of VDR. The concentration of calcitriol, used in antagonist mode, was selected based on dose-response analysis, when the induction of luciferase activity by 50 nM calcitriol (average fold induction 13-fold) was in ascending part of the curve, adjacent to EC_{50} value (2.55 ± 0.64 nM; n = 5; see inserted plots in Figure 3C). We observed very weak (approx. 2-fold) induction of luciferase activity in IZ-VDRE cells by EOs of caraway (250 μg/mL), dill (≥100 μg/mL), lemongrass (10 μg/mL), oregano (50 μg/mL and 100 μg/mL), spearmint (250 μg/mL) and thyme (100 μg/mL) (Figure 3B). Interestingly, we observed augmentation, mostly dose-dependent, of calcitriol-inducible luciferase activity by all EOs. Therefore, to dissect minor effects from real functional potentiation of ligand-inducible activity of VDR, we set cut-off value as 2-fold (200%) augmentation. Then, significant activity was confirmed for EOs of caraway, coriander, dill, ginger, lemongrass, oregano, spearmint, thyme, turmeric and verveine (Figure 3C).

Effects of essential oils on the expression of *CYP24A1*, *TAT* and *PSA* mRNAs in cell lines

In next series of experiments, we tested the effects of EOs on the expression of prototypical target genes of GR, AR and VDR, which are tyrosine aminotransferase (*TAT*), prostate-specific antigen (*PSA*) and vitamin D3 24-hydroxylase (*CYP24A1*), respectively. Experiments were performed only with those EOs, which displayed any activity in reporter gene assays (Figures 1-3).

Expression of *CYP24A1* mRNA was measured in LS180 cells incubated with tested EOs in the absence or in the presence of 50 nM calcitriol. Induction of *CYP24A1* mRNA by calcitriol

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(50 nM) was approx. 72 000-fold. No significant induction was achieved by EOs of dill, caraway, lemongrass, spearmint, oregano and thyme (Figure 4A), implying that the minor effects observed in IZ-VDRE cells on the reporter (Figure 3B) were physiologically irrelevant. On the other hand, calcitriol-mediated induction of *CYP24A1* mRNA was significantly augmented (from +15% to +50%) by EOs of dill, coriander, turmeric, lemongrass, verveine, spearmint, thyme and ginger (Figure 4B), which is consistent with reporter gene data (Figure 3C).

Anti-glucocorticoid effects of EOs were examined in HepG2 cells co-incubated with EOs and DEX (100 nM). Significant diminution of DEX-inducible expression of *TAT* mRNA was observed only for EO of spearmint, but not for EOs of dill, caraway, turmeric, verveine, nutmeg and oregano (Figure 4C), implying physiological insignificance of the data from reporter gene assay (Figure 1C).

Finally, anti-androgen effect of EO of vanilla was tested in 22Rv1 cells, which were co-incubated with DHT (100 nM) and EO of vanilla. DHT itself induced *PSA* mRNA 2-fold, and the co-incubation with EO of vanilla did not influence the effect of DHT (Figure 4D).

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DISCUSSION

Variety of xenobiotics, including environmental pollutants and food-born chemicals, cause endocrine disruption through interactions with steroid and nuclear receptors. In the current paper, we describe the effects of 31 essential oils of culinary herbs and spices on common transcriptional targets for endocrine disruptors. Receptors AR, GR and VDR were selected, as representatives of sex hormone-activated steroid receptor, corticoid hormone-activated steroid receptor and nuclear receptor, respectively. On the one hand, selection of three endocrine disruptor transcriptiona end-points was done to make a study swift and straightforward. On the other hand, this selection may represent certain limitation of the current study, because complex assessment of endocrine disrupting potential for any xenobiotic substance or mixture should address all hormone-responsive targets, i.e. not only AR, VDR and GR, but also RXRs, RARs, TR, PR, ER, MR, PPARs. Also xenobiotic-mediated perturbance of xenosensors (PXR, CAR, AhR) often leads to endocrine disruption. Moderate augmentation of calcitriol-dependent VDR activity was caused by EOs of ginger, thyme, coriander and lemongrass. Mixed anti-glucocorticoid and VDR-stimulatory activities were displayed by EOs of turmeric, oregano, dill, caraway, verveine and spearmint. Remaining 19 EOs were inactive against all receptors under investigation. Analyses of GR, AR and VDR target genes by the means of RT-PCR confirmed VDR-stimulatory, but not anti-glucocorticoid and anti-androgen effects of EOs. A key aspect that makes the study potentially highly relevant is that we used EOs in concentrations really occurring in foods and drinks. Based on the information in cookery books, the concentration of EO in the ingested foods spans at least from 35 μg/mL to 55 μg/mL, which is in the range of concentrations used in the present study [21]. This is, in particular, important and relevant in applications, where EOs are used to modulate intestinal health, as revealed by recent study of Zanthoxylum bungeanum EO on intestinal dysfunction [22]. On the other hand, culinary doses of EOs are not necessarily equal to bioavailable and

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potentially bioactive doses. For instance, many constituents of EOs are volatile chemicals that are directly absorbed in the mouth whereas others may undergo absorption *via* the digestive pathway. Pharmacokinetics of EOs and their constituents is also strongly dependent on the delivery systems, e.g. food matrix, micro-capsules etc. [23]. It should be taken in consideration that EOs are multicomponent mixtures of biologically active compounds, therefore, overall effects of EO comprise activities of its individual constituents. Indeed, in our recent study of EOs´ effects on AhR, we observed differential effects of individual constituents as compared to their parental EOs. EOs of basil and tarragon contain similar amount of estragole (about 75%). While EO of tarragon, as well as estragole, antagonized AhR, EO of basil was inactive at AhR [14]. This phenomenon might be explained by combined effects of estragole and other constituents of both EOs. The direct molecular effects of individual compounds on particular receptor may combine full agonist, partial agonist, inverse agonist and antagonist effects. In addition, the effects may be indirect or may involve off-targets. In case of mixtures, resulting activities may be mutually additive, synergistic, opposite or counteracting. The recent discovery of co-binding of two inactive compounds to PXR, leading to synergistic activation of the receptor, a phenomenon called the "formation of a supramolecular ligand", further makes situation more complicated [24]. Analogically, binding of two indole molecules to human AhR, thereby mimicking the binding of indirubin, was recently described [25]. On the other hand, unlike steroid and nuclear receptors, both PXR and AhR are so called high-capacity / low-specificity receptors, allowing such a molecular mechanism.

There exist multiple cross-talks between signaling pathways of steroid and nuclear receptors. Functional cross-talks, with physiological or patho-physiological consequences were described for PXR-GR [26], RXR-GR [27], AhR-GR [28], AhR-PXR [29], PXR-VDR [30], AhR-ER [31] and many other pairs and cascades. These cross-talks may also apply for some

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data in the present paper. For instance, we show here that EOs of caraway, dill, nutmeg, oregano, spearmint, turmeric and verveine display anti-glucocorticoid effects in AZ-GR reporter gene cells (Figure 1C). In our recent paper, we described that EOs of caraway, dill, nutmeg, oregano, spearmint and turmeric have full or partial agonist activities on AhR [14]. Since AhR ligands such as dioxin were found to suppress dexamethasone-inducible expression of GR target gene TAT [28], the plausible explanation for anti-glucocorticoid effects of EOs observed here, may be the activation of AhR by these EOs.

In conclusion, while we observed minor effects of several EOs on transcriptional activities of GR, AR and VDR, the toxicological significance is very low. Hence, 31 EOs of culinary herbs and spices may be considered safe, in terms of endocrine disruption involving receptors GR, AR and VDR.

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ABBREVIATIONS

AhR, Aryl Hydrocarbon Receptor; AR, Androgen Receptor; CAR; Constitutive Androstane Receptor; DEX, Dexamethasone; DHT, 5α-dihydrotestosterone; EO, Essential Oil; GR, ER, Estrogen Receptor; Glucocorticoid Receptor; MR, Mineralocorticoid Receptor; PPAR, Peroxisome Proliferator-Activated Receptor; PR, Progesterone Receptor; PXR, Pregnane X Receptor; RAR, Retinoic Acid Receptor; RXR, Retinoid X Receptor; VDR, Vitamin D Receptor

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FIGURE LEGENDS

Figure 1. Transcriptional activity of glucocorticoid receptor GR by essential oils. AZ-GR cells were incubated for 24 h with vehicle (EtOH; 0.1% v/v), dexamethasone (DEX; 100 nM) and EOs in concentrations ranging from 0.01 μg/mL to 250 μg/mL. All experiments were performed in three consecutive passages of AZ-GR cells. # = not tested. * = value significantly different from control cells ($p<0.05$). ***Panel A: Cytotoxicity assay – MTT test.*** The data are mean from experiments from three consecutive cell passages and are expressed as a percentage of viability of control cells. ***Panel B: Agonist mode.*** Incubations were carried out in the absence of dexamethasone. Cells were lysed and luciferase activity was measured. Data are expressed as a fold induction of luciferase activity over control cells and they are the mean ± SD from a representative experiment (cell passage). ***Panel C: Antagonist mode.*** Incubations were carried out in the presence of 100 nM dexamethasone. Cells were lysed and luciferase activity was measured. Data are expressed as a percentage of the activation attained by 100 nM DEX and they are the mean ± SD from a representative experiment (cell passage). Inserted plots (bottom right) show dose-response effect of DEX.

Figure 2. Transcriptional activity of androgen receptor AR by essential oils. AIZ-AR cells were incubated for 24 h with vehicle (EtOH; 0.1% v/v), 5α-dihydrotestosterone (DHT; 100 nM) and EOs in concentrations ranging from 0.01 μg/mL to 250 μg/mL. All experiments were performed in three consecutive passages of AIZ-AR cells. # = not tested. * = value significantly different from control cells ($p<0.05$). ***Panel A: Cytotoxicity assay – MTT test.*** The data are mean from experiments from three consecutive cell passages and are expressed as a percentage of viability of control cells. ***Panel B: Agonist mode.*** Incubations were carried out in the absence of DHT. Cells were lysed and luciferase activity was measured. Data are expressed as a fold induction of luciferase activity over control cells and they are the mean ±

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SD from a representative experiment (cell passage). ***Panel C: Antagonist mode.*** Incubations were carried out in the presence of 100 nM DHT. Cells were lysed and luciferase activity was measured. Data are expressed as a percentage of the activation attained by 100 nM DHT and they are the mean ± SD from a representative experiment (cell passage). Inserted plots (bottom right) show dose-response effect of DHT.

Figure 3. Transcriptional activity of vitamin D receptor VDR by essential oils. IZ-VDRE cells were incubated for 24 h with vehicle (EtOH; 0.1% v/v), 1α,25-dihydroxyvitamin D3 (calcitriol; 50 nM) and EOs in concentrations ranging from 0.01 μg/mL to 250 μg/mL. All experiments were performed in three consecutive passages of IZ-VDRE cells. # = not tested. * = value significantly different from control cells ($p<0.05$). ***Panel A: Cytotoxicity assay – MTT test.*** The data are mean from experiments from three consecutive cell passages and are expressed as a percentage of viability of control cells. ***Panel B: Agonist mode.*** Incubations were carried out in the absence of calcitriol. Cells were lysed and luciferase activity was measured. Data are expressed as a fold induction of luciferase activity over control cells and they are the mean ± SD from a representative experiment (cell passage). ***Panel C: Antagonist mode.*** Incubations were carried out in the presence of 50 nM calcitriol. Cells were lysed and luciferase activity was measured. Data are expressed as a percentage of the activation attained by 50 nM calcitriol and they are the mean ± SD from a representative experiment (cell passage). Inserted plots (bottom right) show dose-response effect of calcitriol.

Figure 4. Effects of essential oils on the expression of *CYP24A1, PSA* and *TAT* mRNA in cell lines. ***Panel A,B: Expression of CYP24A1.*** The LS180 cells were incubated with vehicle and EOs (single concentration), in the absence (Panel A) and the presence (Panel B) of 50 nM calcitriol. ***Panel C: Expression of TAT.*** The HepG2 cells were incubated with vehicle and

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EOs (single concentration), in the presence of 100 nM dexamethasone. ***Panel D: Expression of PSA.*** The 22Rv1 cells were incubated with vehicle and EOs (single concentration), in the presence of 100 nM 5α-dihydrotestosterone (DHT). Incubations were carried out in triplicates, and the duration of treatment was 24 h. All experiments were performed in two consecutive cell passages. The vehicle was EtOH (0.1% v/v; UT = untreated). The levels of *CYP24A1, TAT* and *PSA* mRNAs were determined by RT-PCR and the data were normalized to *GAPDH* mRNA level. Data are mean ± SD. * = value significantly different from control cells ($p<0.05$).

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HeLa cell line - cytotoxicity assay

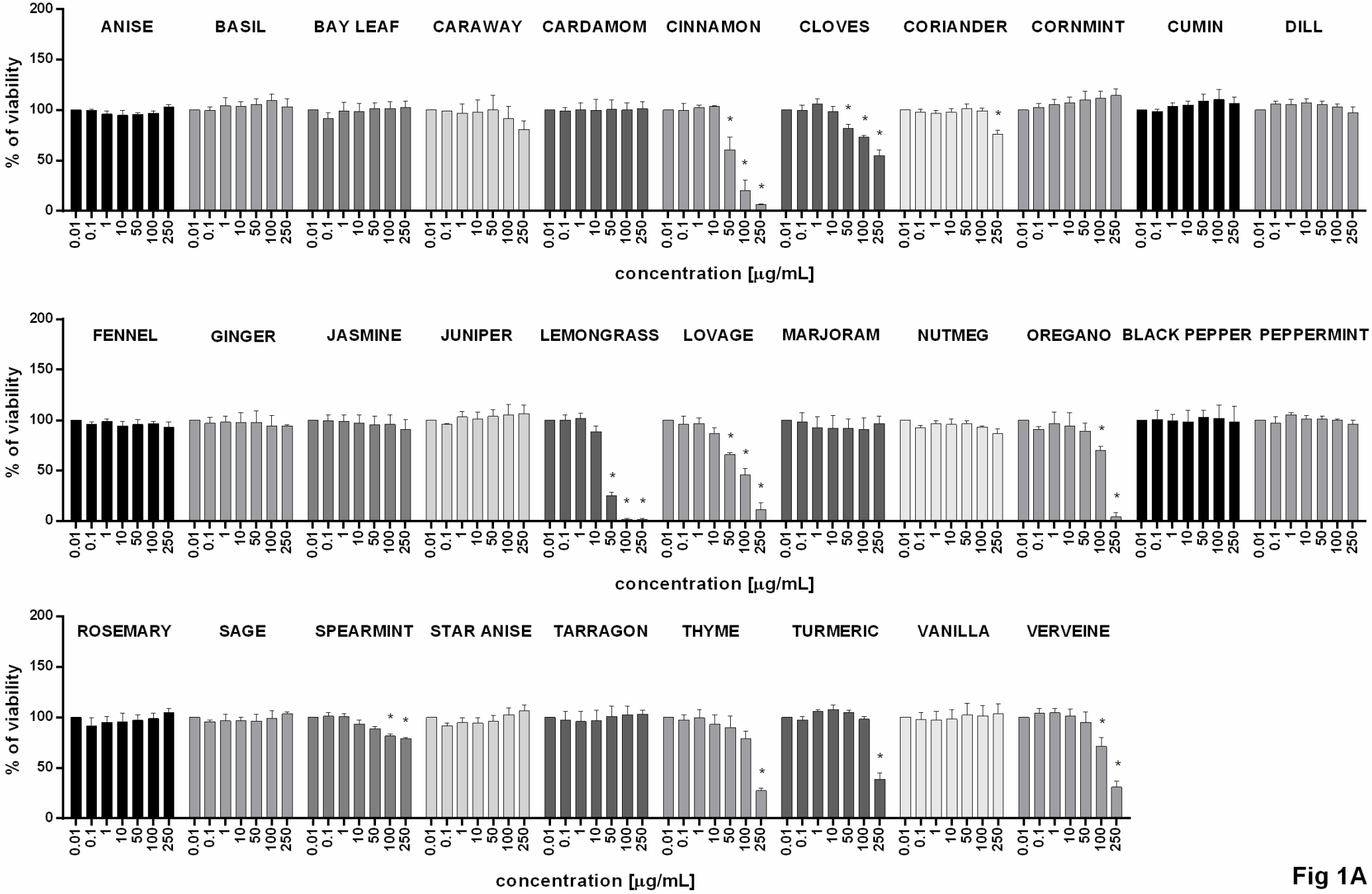

Fig 1A

AZ-GR agonist mode

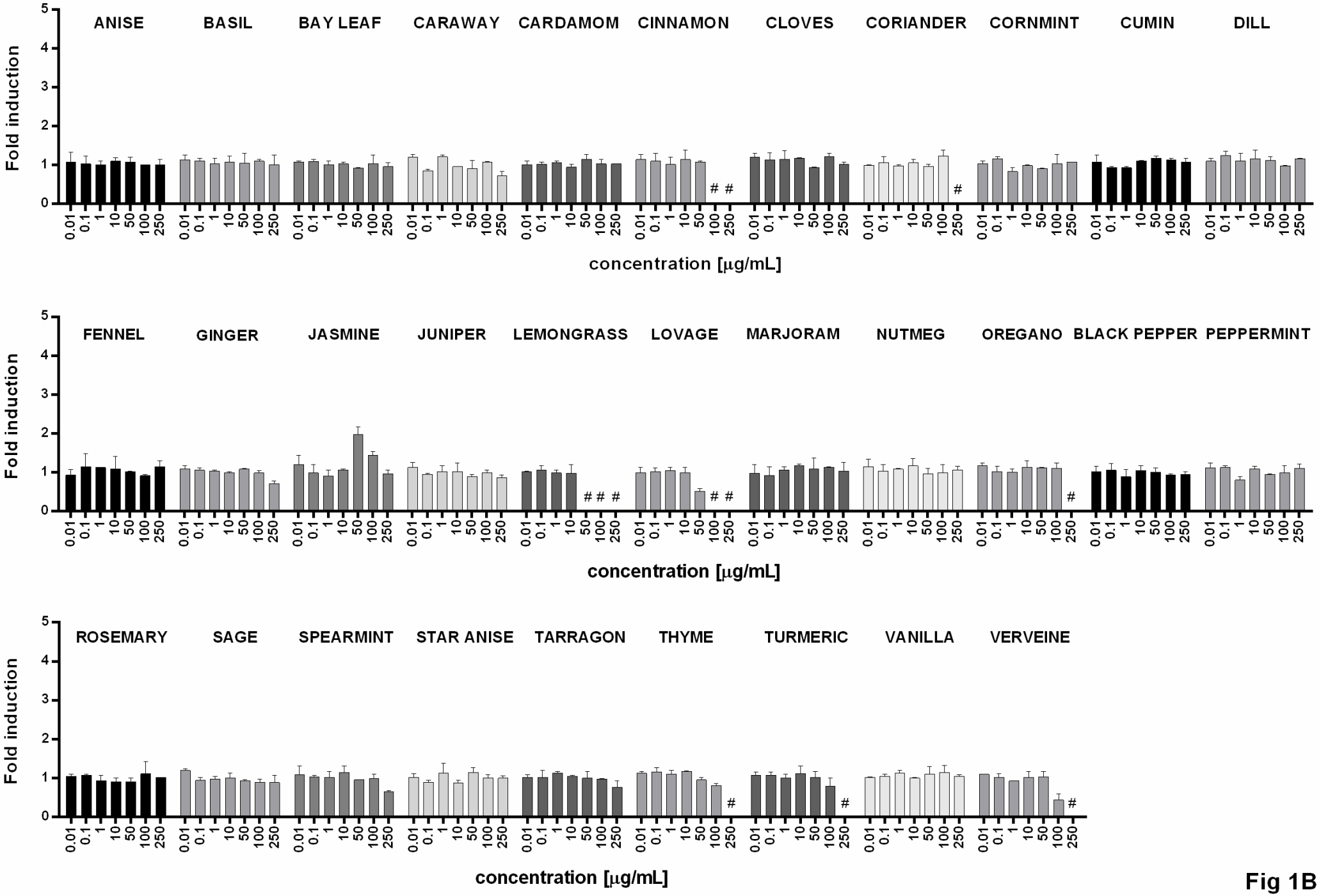

Fig 1B

AZ-GR antagonist mode (100 nM dexamethasone)

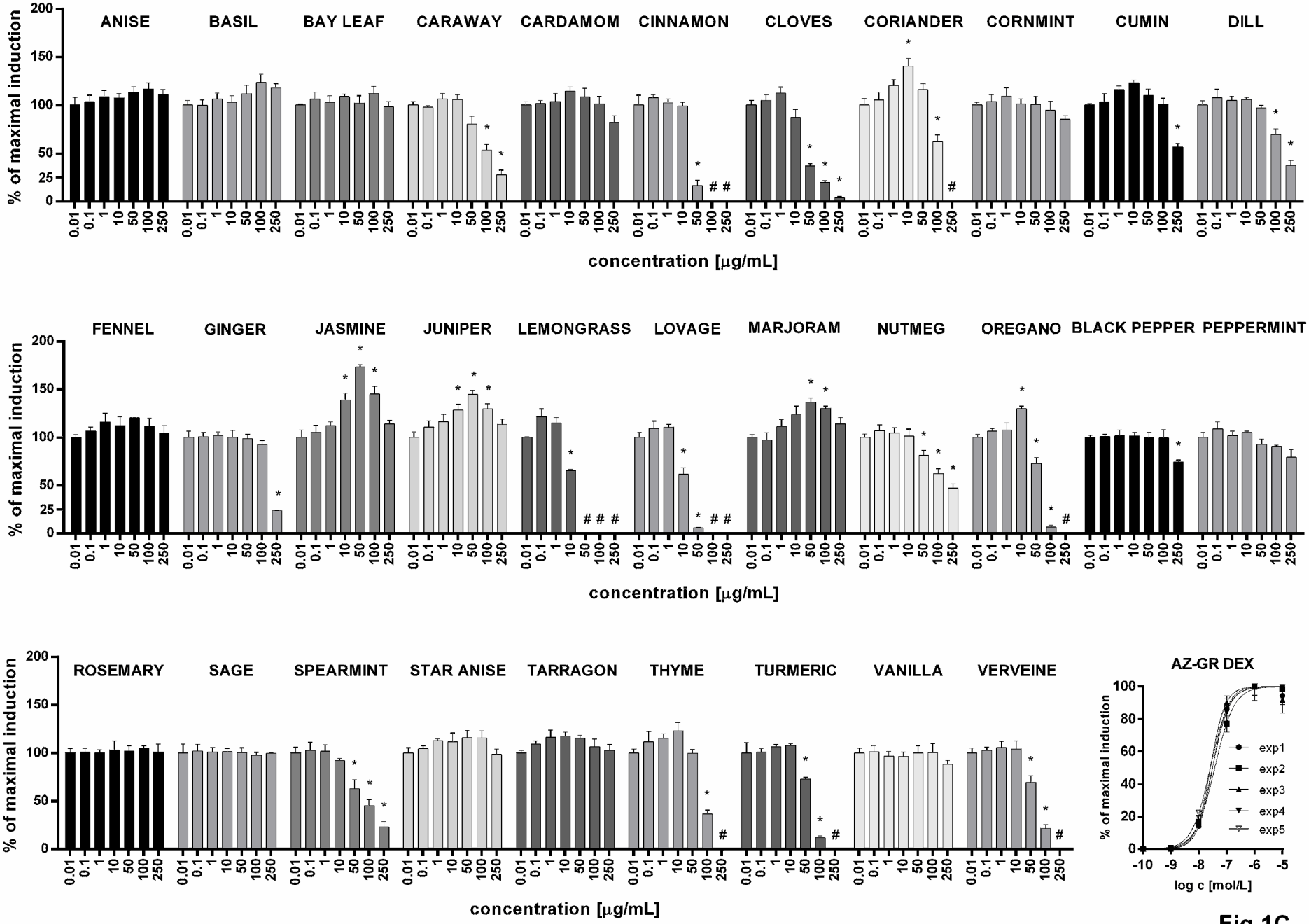

Fig 1C

22Rv1 cell line - cytotoxicity assay

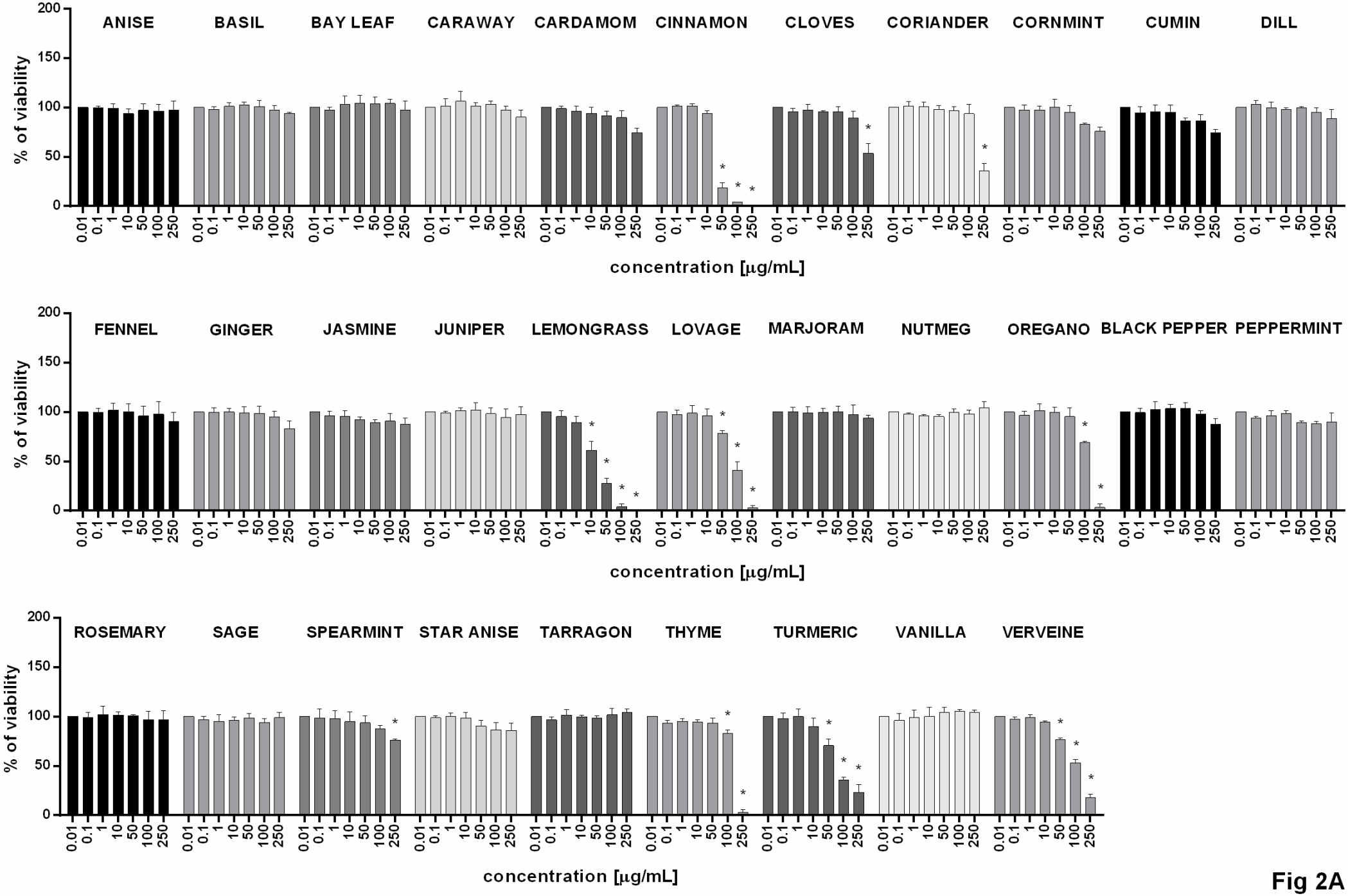

Fig 2A

AIZ-AR agonist mode

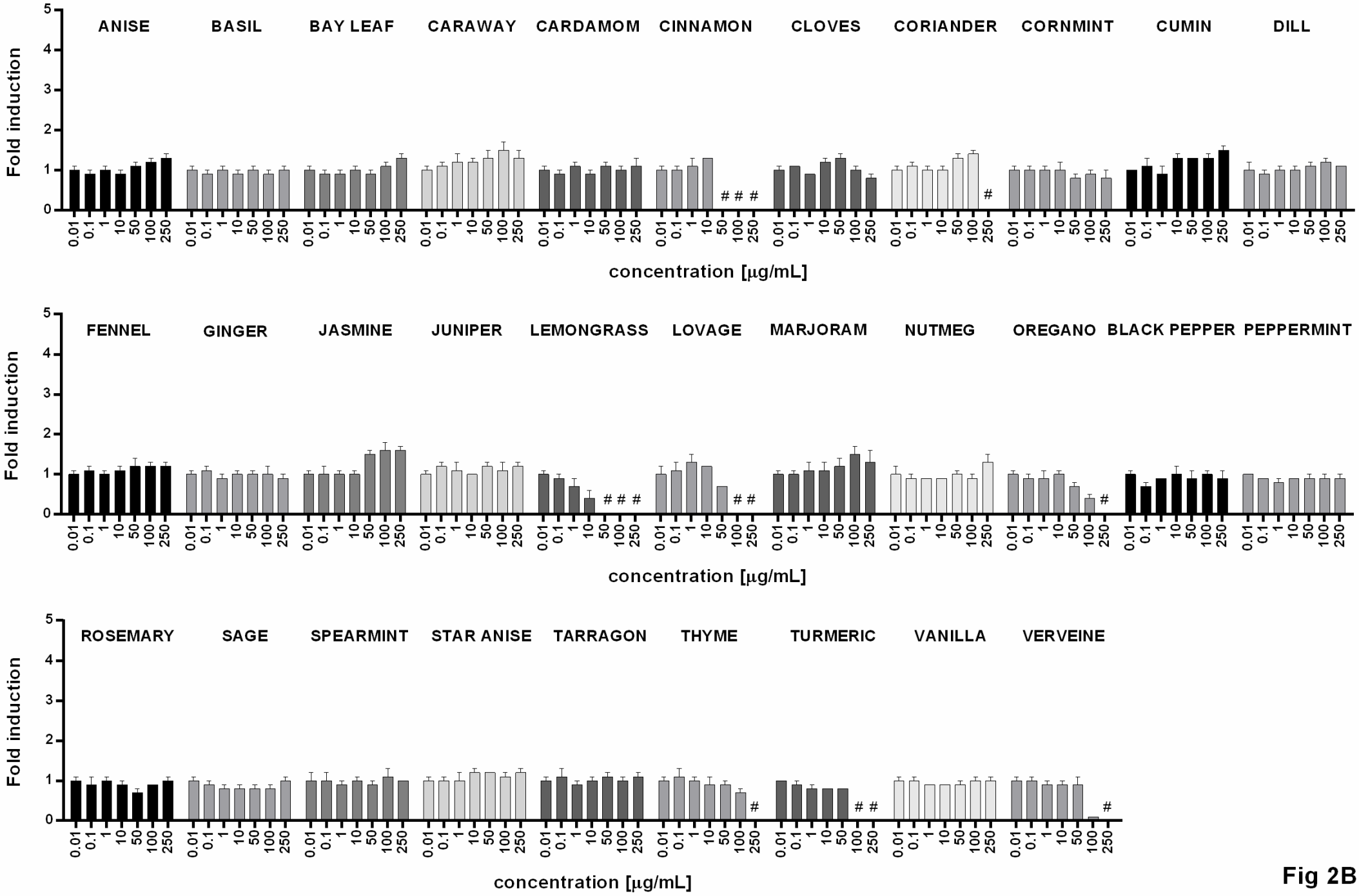

Fig 2B

AIZ-AR antagonist mode (100 nM 5α-dihydrotestosterone)

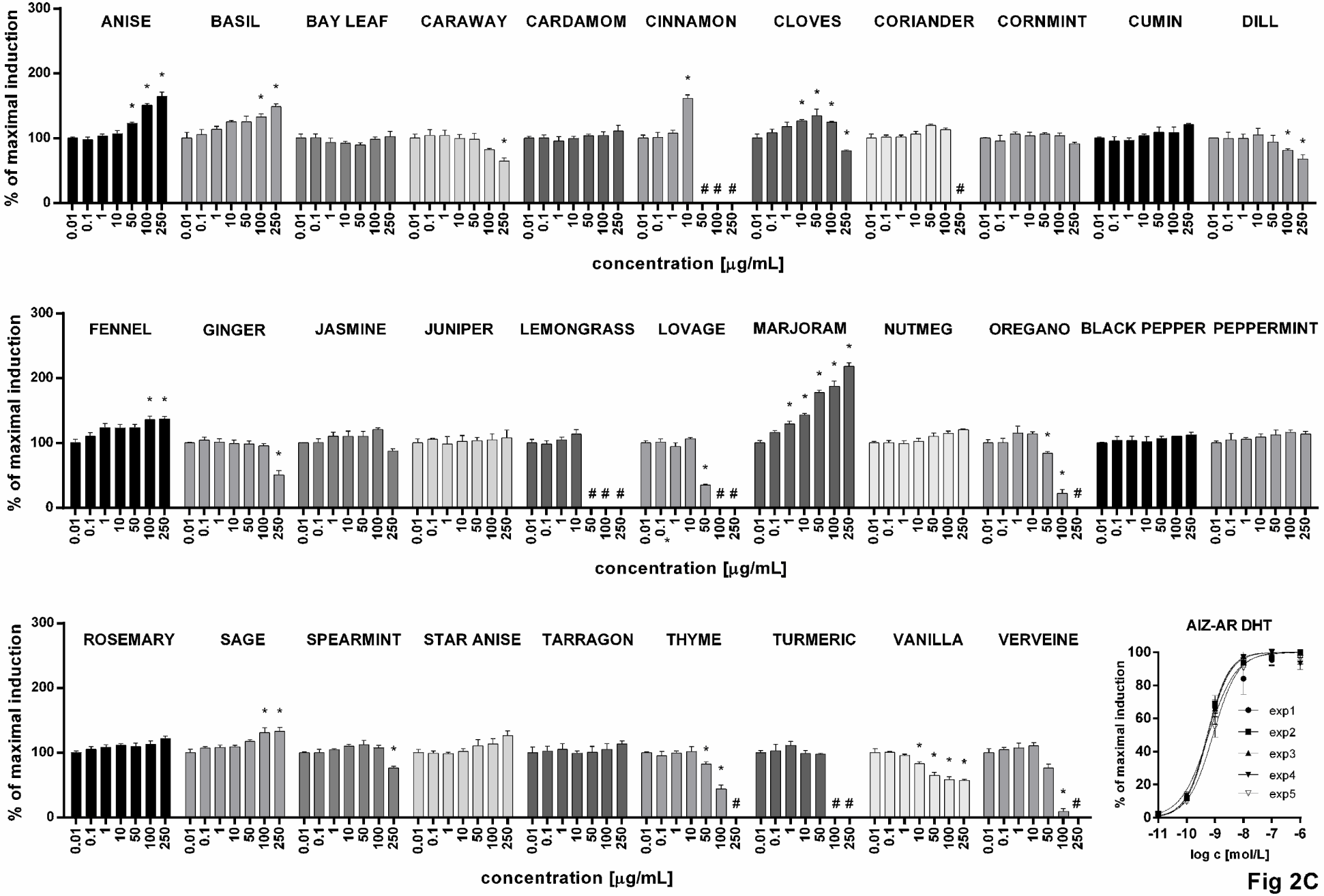

Fig 2C

LS 180 cell line - cytotoxicity assay

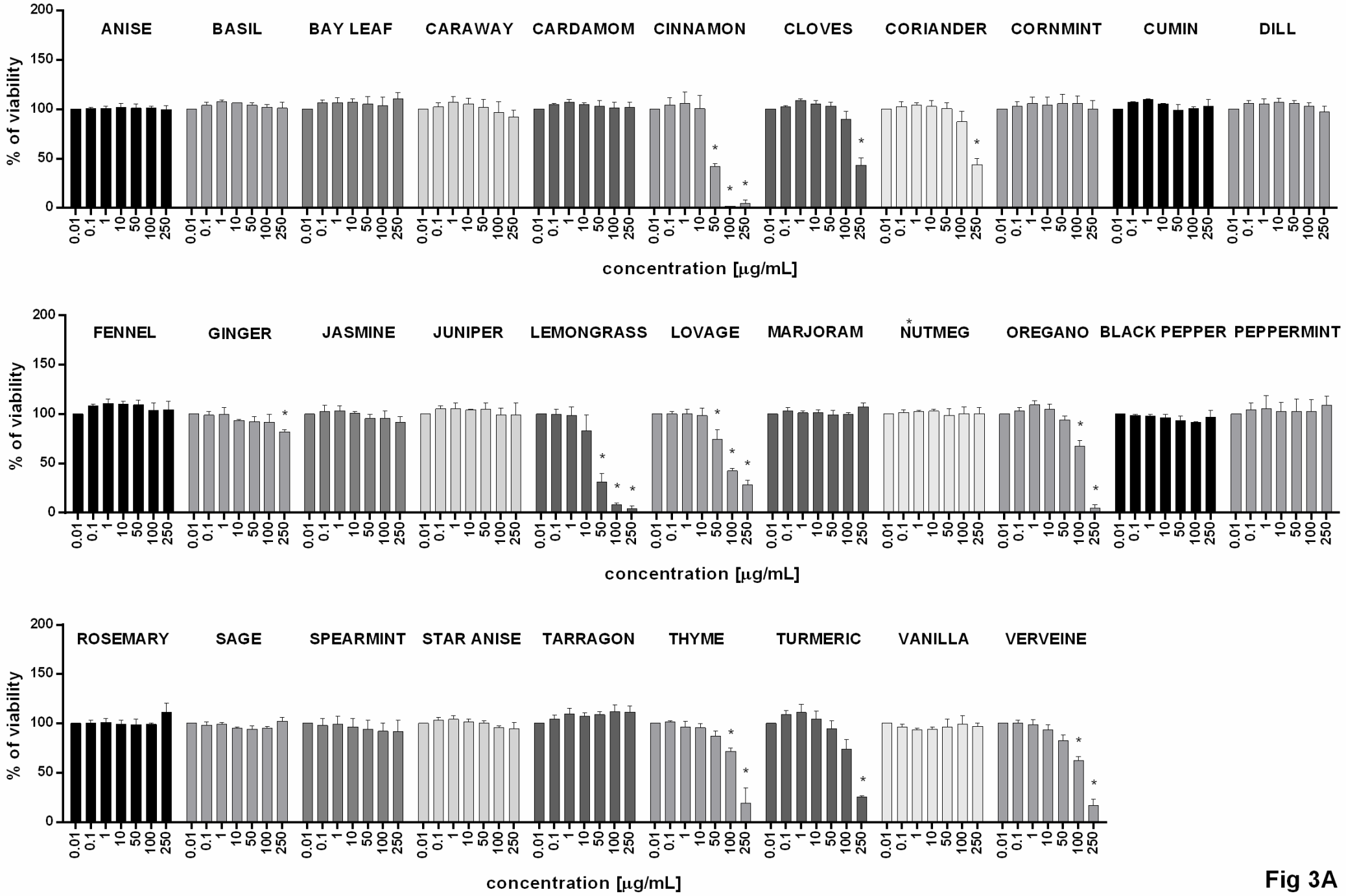

Fig 3A

IZ-VDRE agonist mode

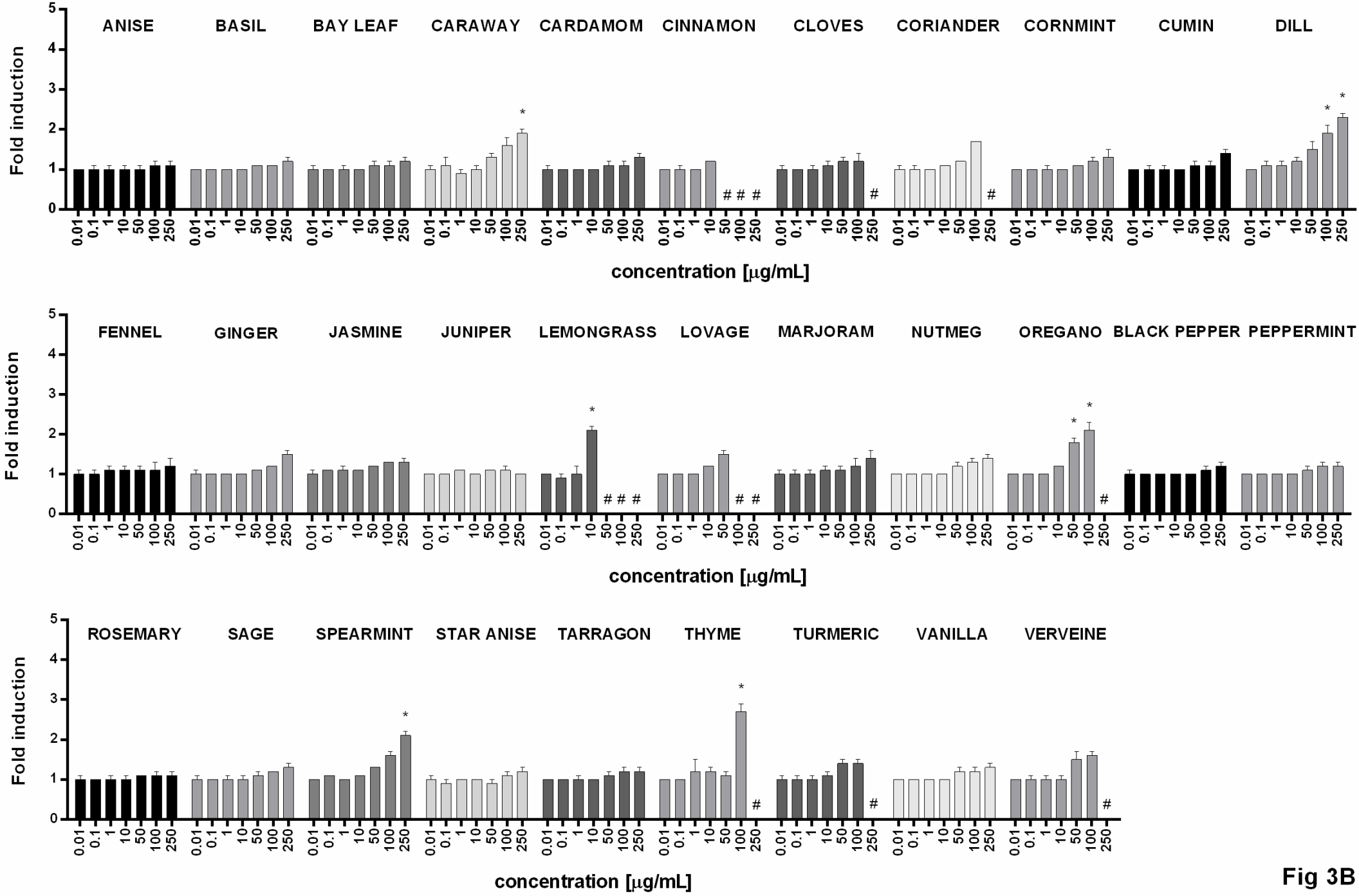

Fig 3B

IZ-VDRE antagonist mode (50 nM calcitriol)

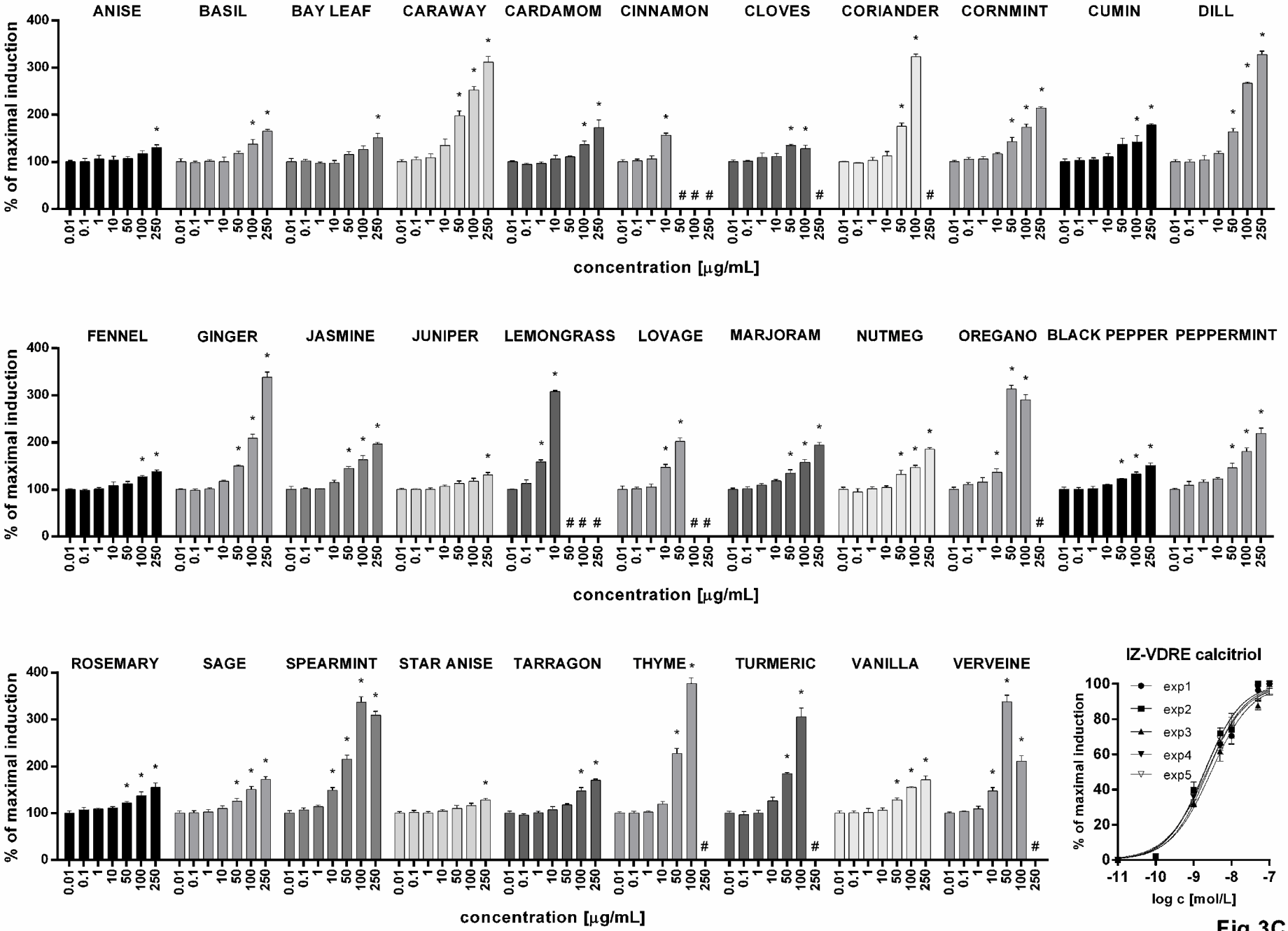

Fig 3C

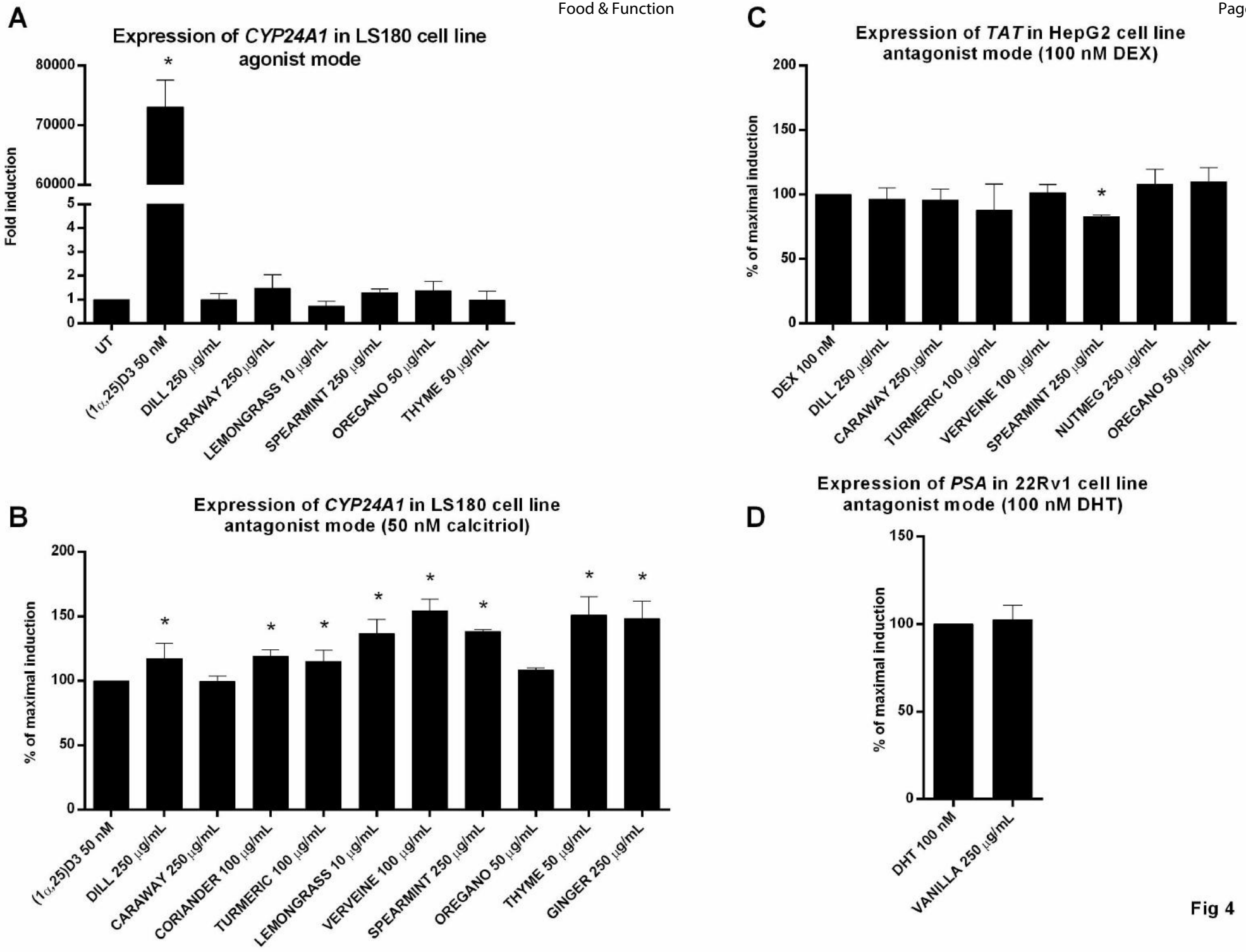

Fig 4

ESSENTIAL OILS OF CULINARY HERBS AND SPECIES INFLUENCE TRANSCRIPTIONAL ACTIVITIES OF NUCLEAR RECEPTOR VDR AND STEROID HORMONES RECEPTORS AR AND GR

Figure 5

Table 1: Primer sequences with appropriate Universal Probes Library (UPL) numbers.

Gene symbol	Forward primer sequence	Reverse primer sequence	UPL no.
GAPDH	CTCTGCTCCTCCTGTTCGAC	ACGACCAAATCCGTTGACTC	60
CYP24A1	TCATCATGGCCATCAAAACA	GCAGCTCGACTGGAGTGAC	88
PSA (*KLK3*)	GTGCTTGTGGCCTCTCGT	CAGCAAGATCACGCTTTTGT	44
TAT	GCACCCCTAGAAGCTAAGGAC	CAGGTCTTGGAACCAGGATG	37