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Essential oil from *Cymbopogon flexuosus* as the potential inhibitor for HSP90

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ABSTRACT

The essential oil of *Cymbopogon flexuosus* or lemongrass oil (LO) is reported to have antibacterial, antifungal and anticancerous effects. HSP90 is one of the major chaperones responsible for the proper folding of cancer proteins. In this paper we show that the essential oil of *C. flexuosus* significantly suppresses the HSP90 gene expression. The cytotoxicity of the compounds was tested by MTT assay and the gene expression studies were carried out using HEK-293 and MCF-7 cells. Also we tested the efficacy of the major component of this essential oil viz. citral and geraniol in inhibiting the HSP90 expression. The oil was found to be more cytotoxic to MCF-7 cells with different IC_{50} values for the oil (69.33 µg/mL), citral (140.7 µg/mL) and geraniol (117 µg/mL). The fold change of expression was calculated by RT-qPCR using ΔΔCt ($2^{-\Delta\Delta Ct}$) method and it was 0.1 and 0.03 in MCF-7 cells at 80 µg/mL and 160 µg/mL of LO. Western blot results showed suppression of HSP90 protein expression and HSP90 – ATPase activity inhibition was also observed using LO. This study shows the anticancer mechanism exhibited by the essential oil of *C. flexuosus* is by the inhibition of the important chaperone protein HSP90.

1. Introduction

Breast cancer was found to be the second highest cause of death in women in USA and as of March 2017 statistics, more than 3.1 million women with a history of breast cancer [1]. Breast cancer is classified into subtypes based on the receptors expressed on the tumour cells viz. estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth receptor (HER 2) [2]. The triple negative subtype had the minimum survival. MCF-7 cells are extensively used human breast adenocarcinoma cells with the useful characteristics similar to mammary epithelium, having the capability to process estrogen (ER positive) [3]. The estrogen induces the proliferation of MCF-7 and the therapy for breast cancer targets mainly the blocking of estrogen action [4]. The anti-estrogens are used as therapeutics against the ER + ve tumours have the inhibitory effects on MCF-7 [5]. Estrogen binds to the DNA and modulates the gene expression of ER [6]. Selective estrogen receptor modulators (SERMS) i.e. tamoxifen compete with estrogen for ER binding and thus the antiestrogen therapy is one of the major therapies in ER positive breast cancer [7].

The treatment with anti-estrogen drugs like tamoxifen is the most preferred choice for the ER positive breast cancers. The resistance developed against the antiestrogen therapy is becoming a major problem in treating the estrogen dependent breast cancers. The de novo resistance is the complete absence of the estrogen receptor function or it may be the acquired resistance by the unresponsiveness of the hormone receptors to the anti-estrogens [8]. Different compositions consisting of the admixture of different essential oils and pharmaceutically acceptable carrier has been reported to be useful in the treatment of breast cancer [9]. The anti-proliferative and anti-estrogenic properties of the essential oils could be useful as alternative therapy in treating estrogen dependent cancers. Though there are monoterpenoids like linalool, d-limonene and α-terpineol have been studied for potential treatment for breast cancer, there are no reports about their anti-estrogenic mechanism.

The essential oil of *C. flexuosus* is reported to inhibit the proliferation of various cancer cells [10]. A component of LO i.e. citral, an important monoterpene and a flavouring agent is reported to inhibit the proliferation of human and murine cancer cell lines A2780, ECC-1,

Abbreviations: DEPC, diethyl pyrocarbonate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ER, estrogen receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GC–MS, gas chromatography–mass spectrometry; HER 2, human epidermal growth receptor 2; HPLC, high performance liquid chromatography; HSP90, heat shock protein 90; LO, lemongrass oil; MTT, 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide; PR, progesterone receptor; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; SERMS, selective estrogen receptor modulators

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OVCAR-3, SKOV-3, ID8 and MOVCAR [11]. Citral is reported to induce benign and atypical prostate hyperplasia [12] and also the hormone sensitive tissue responsiveness was reported by its anti-estrogenic actions [13]. One of the precursors of citral i.e. geraniol was also found to induce gynacomastia in man following its use [14]. In another study both citral and geraniol, did not show estrogenic or antiestrogenic actions at lower concentrations. Also these compounds did not show any estrogenic response in overiectemized mice. However at higher concentrations both citral and geraniol showed anti-estrogenic activity in estrogen responsive human cell line Ishikawa var – I [15]. Citral is also reported to inhibit the proliferation and also inducing apoptosis in estrogen receptor positive MCF-7 cells [16].

There are other compounds isolated from the natural sources and used in folk remedy reported to induce apoptosis in MCF-7 cells. Farnesiferol-C which was isolated from *Ferula asafoetida* (a type of coumarins) is induces cell cycle arrest and apoptosis in MCF-7 cells [17]. Also the naturally occurring secondary metabolites viz. depsidones isolated from marine fungus also have shown to inhibit tumour cell proliferation in T47D breast tumour cells [18]. These studies show the anticancer potential of the compounds used in folk medicines.

Though the anticancer properties are reported for the lemongrass essential oil, we report the anticancer mechanism by the inhibition of the important chaperone involved in oncogenic pathways viz. HSP90 gene and protein. The present study gives an understanding of the effect of lemongrass oil and its constituents viz. citral and geraniol on gene and protein expression of HSP90 in MCF-7 cells in comparison with normal HEK-293 cells. Also here we study the functional inhibition of HSP90-ATPase activity using pure HSP90 protein. To separate the components of LO and evaluate the mechanism of binding of those components into the N-terminal or C-terminal domains of HSP90 to study the interactions would be the future perspective for this study

2. Materials and method

MCF-7 (HTB22) and HEK-293 (CRL1573) were obtained from ATCC. The essential oil of *C. flexuosus* (LO) was obtained from Perfect Herbals and Oils, Madhya Pradesh, India. Citral (95%) and geraniol (98%) were obtained from Sigma, India. The essential oil, citral and geraniol were analysed using the newly developed HPLC method in our laboratory and also by GC–MS [19]. The DMEM medium, FBS, penicillin G, streptomycin were obtained from Invitrogen, India. MTT powder was obtained from Sigma, India. The solution is filtered through a 0.2 μ m filter and stored at 2–8 °C for frequent use or frozen for extended periods. DMSO and DEPC water, trizol and isopropanol were obtained from Sigma, India. Tecan Plate reader was from Tecan, Switzerland. The chloroform and ethanol were obtained from Ranchem. PBS required for cell culture was obtained from Himedialabs, India. 1.5 and 2 mL vials were obtained from Eppendorf and 15 mL falcon tubes were obtained from Tarsons Ltd. The vials and tubes were washed with DEPC treated water, autoclaved and oven dried. Human HSP90 protein was obtained from Stressmarq Biosciences, Canada. ATP– Sodium salt was obtained from Sigma, India. Other reagents used in the HSP90 – ATPase inhibition experiement were of analytical grade.

The rabbit polyclonal antibody against HSP90 (sc-7947) was obtained from Santa-Cruz, Biotechnology, USA. The secondary anti-rabbit antibodies were from Invitrogen. The beta actin primary antibody (MA5-15739) and the anti-mouse secondary antibodies were also procured from Invitrogen. The SDS-PAGE and western blot analysis were done using the buffers from Invitrogen and ECL kit from Thermo Scientific-Pierce.

2.1. Cytotoxicity study

Cytotoxicity of LO and citral was tested on breast cancer cell lines MCF-7 using MTT assay. Cells were collected when they reach about 70–80% confluency. Cells were checked for viability using Trypan blue staining and centrifuged. The wells were seeded at 50,000 cells / well of MCF-7 in a 96 well plate and incubated for 24 h at 37 °C, 5% CO_2 incubator. The essential oil, citral and geraniol were added from 5 to 320 μg/mL concentration in DMEM without FBS and incubated for 24 h. After incubation with test samples, filtered MTT (5 mg/10 mL of MTT in 1X PBS) was added to the respective wells (50 μg /well) and incubated for 3–4 h. After incubation with MTT reagent, the MTT reagent was discarded by pipetting without disturbing cells and 100 μL of DMSO was added to rapidly solubilize the formazan. The absorbance was measured at 590 nm.

Table 1
Primers used for the RTPCR reaction.

Gene	Primer pair	Sequence (5' → 3')	Tm	GC %	Expected product size (bp)
HSP90	FP	TGGACAGCAAACATGGAGAG	57.3	50	204
	RP	AGACAGGAGCGCAGTTTCAT	57.3	50	
GAPDH	FP	CGACCACTTTGTCAAGCTCA	58.4	50	238
	RP	CCCCTCTTCAAGGGGTCTAC	58.8	60	

2.2. Gene selection and primer design

The GAPDH was used as housekeeping gene for HEK-293 and MCF-7 cell lines. HEK-293 was used as control cell line for the comparison of gene expression. Primers were designed for GAPDH gene (NG_007073) and HSP90 gene (NM_001017963.2) using Primer– 3 and synthesized for GAPDH and HSP90 at Eurofins Genomics, India. The details of the primers were as tabulated in Table 1.

2.3. RNA isolation

Both MCF-7 and HEK-293 cultured in DMEM medium and subjected to different treatments were washed twice with PBS and to the adherent cells 2 mL of Trizol (per T25 flask) was added and transferred to a falcon tube and vortexed. Samples were allowed to stand for 5 min at room temperature. To this 0.2 mL of chloroform per 1 mL of Trizol was used. The tube was shaken vigorously for 15 s. The tube was allowed to stand at room temperature for 5 min. The resulting mixture was centrifuged at 10,000*g* for 15 min at 4 °C. The colourless upper aqueous phase was transferred to a new clean tube. To this 0.5 mL of isopropanol was added per 1 mL of Trizol used. The mixture was mixed gently and incubated at room temperature for 5 min and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded and the RNA pellet was washed by adding 1 m L of 70% ethanol and mixed properly. The suspension was centrifuged for 5 min at 14,000 rpm at 4 °C and the supernatant was discarded. The pellet was dried by incubating in a dry bath for 5 min at 55 °C. The pellet was then resuspended in 25 μL of DEPC treated water (Table 2).

2.4. cDNA synthesis and semiquantitative RT-PCR

A semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Techne Prime system to determine the levels of HSP90 and GAPDHmRNA expressions. The cDNA was synthesized from 2 μg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20 μL and cDNA synthesis was performed at 42 °C for 60 min, followed by RT inactivation at 85 °C for 5 min. The PCR mixture (final volume of 20 μL) contained 1 μL of cDNA, 10 μL of Red Taq Master Mix 2x (Amplicon) and 1 μM of each complementary primer specific for HSP90 and GAPDH (internal control) sequence (Table 1). The samples were denatured at 94 °C for 5 min, and amplified using 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and