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Effect of Plant Extracts on Innate Immunity of Skin Cells: Investigations on Human Keratinocyte Cell Lines

Research Article

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Abstract

Stresses caused by many factors including pathogenic bacteria result in inflamatory diseases and they are considered as a major source of skin cancers and premature ageing.

Plants growing in semi-arid environments and deserts present the astonishing capacity to survive extreme conditions of drought and high temperatures. The objective of this study is to investigate the effects of extracts from two endemic plants from Saudi Arabia, namly C. myrrha and Rhamnus frangula, on the immune response of human keratinocyte cell lines (HaCaT).

Our major findings are i) the extracts from both plants exhibit a significant anti-oxidant activity. The concentrations used are below the limit value of toxicity. ii) These extracts stimulate the innate immune response in HaCaT cells and iii) treatments with these extracts are not harmful to the natural cutaneous microflora.

Together these findings are highly promising with regards to skin protection.
It was in this work investigate the impact of plant extracts on the immune response of human keratinocyte cell lines (HaCaT). In this study, the elicitor activity of the extracts obtained from two endemic plants Arabia *C. myrrha* and *R. frangula* was evaluated. These plants were collected and provided by Dr. Samiah Hamad Al-Mijalli from (Saudia Arabia). The antioxidant activity of *C. myrrha* and *R. frangula* extracts was assessed for its ability to scavenge free radicals and to protect human keratinocytes from oxidative stress. Furthermore, the impact of such treatment was evaluated on both HaCaT cell viability and on the growth/proliferation of the natural cutaneaous microbiota.

Keywords: Bacteria; *Commiphora Myrrha*; Immunity; *Rhamnus Frangula*; *Skin*.

Abbreviations: A Chromophore Termed Bathocuproine (BATO); Commiphora myrrha (*C. myrrha*); Dulbecco's modified Eagle's medium (DMEM); Human keratinocyte cell lines (HaCaT); Oxygen Radical Absorbance Capacity (ORAC); Potent oxidant, 2,2_- azobis (2-amidinopropane) dihydrochlorid (AAPH); Rhamnus frangula (*R. frangula*); Reactive oxygen species (ROS); Total Anti-oxidant Capacity (TAC).

Introduction

The skin is a natural barrier between the body and the environment and is colonized by a large number of microorganisms. Environmental aggressions such as UV exposure, tobacco and pathogenic micro-organisms are known to have deleterious effects on skin ultrastructure thus resulting in a loss of its protective function. Chronic or excessive environmental stresses lead to persistent inflamatory disease considered as a major source of skin cancers and premature ageing [4]. As a result of these various aggressions, the skin is constantly exposed to reactive oxygen species (ROS) production causing the so-called oxidative stress. ROS

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are highly toxic to the skin layers, and ROS-mediated oxidative damage involves many biological molecules such as DNA modification (e.g., changes in DNA methylation), lipid peroxidation and secretion of inflammatory cytokines [7].

It has long been recognized that many naturally-occurring substances in plants have antioxidant activities [2]. Multipotent antioxidants are molecules that in addition to antioxidant activity possess pharmacological activitues. There are numerous examples of multipotent antioxidants among natural products. Besides their antioxidant properties, some natural antioxidants inhibit platelet-aggregation or display antineoplastic, anti-inflammatory and other activities [3]. The antioxidant activity of plant extracts is of particular interest both because of their beneficial physiological activity on human cells and the potential they have to replace synthetic antioxidants [6].

The objective of the present study is to take advantages of two endemic plants of Saudi Arabia and native to desert area. Such plants are known to be adapted to extreme environmental conditions and develop fascinating strategies to cope with both drought stress and extreme temperature. As a consequence, we postulated that these unexplored plants might be a source of new natural products that can potentially be used in cosmetic and dermatology.

Material and Methods

Cells Culture

HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco®) upplemented with 10% fetal bovin serum (FBS south american, Gibco®) and 1% penicillin–streptomycin at 37°C in a 5% CO_2 humidified atmosphere.

Briefly, the skin specimens were washed with DMEM, trypsinized (0.25% trypsin) at 37°C for 3min to detach. Cells were collected and placed onto 96. Keratinocytes were used for experiments at 60–80% of confluence.

Before all measurements/experiments, HaCaT cells were starved in DMEM without addition of FBS for 24 h. The starvation conditions were necessary to uniform the experiments and exclude any undesired interference of serum components with cell response to the stimulus.

ROS production by HaCaT cells in response to plant extracts

In situ detection of general ROS was performed by staining HaCaT cells cells with the probe CM-H_2DCFDA , which is nonfluorescent in its reduced form. In living cells, cell esterases convert CM-H2DCFDA into 2,7-dichlorodihydrofluorescein diacetate, which is subsequently oxidized by ROS into the fluorescent form 2,7-dichlorofluorescein in the presence of ROS. 2 104, maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS under 5% CO_2 at 37°C, were seeded in 96-well plates and grown for 24 h to let them attach to the bottom of the well. The cells were then incubated with different concentrations of hot water plant extracts at concentrations ranging from 0.031 mg.mL^{-1} to 4 mg.mL^{-1} to *R. frangula* and *C. myrrha* for 45 min, and then incubated with 100µl L of 5 µM CM-H_2DCFDA. The fluorescence of the samples was thus measured at 535nm (excitation 490 nm), using the instrument (Flexstation 3, PRIMACEN), and from each measure the cell auto-fluorescence value was subtracted.

Viability and proliferation of HaCaT cells in response to plant extracts

Effects of stress exposures and their combination with hot water plants extracts on HaCaT cells growth and vitality as well as membrane integrity were assessed after 24 h by standard assays. The keratinocyte proliferation and cell survival was determined by MTT (3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazolium bromide) colorimetric assay described elsewhere. The vitality and growth rate determinations were performed in triplicate for each experimental condition.

TAC and ORAC Assays

Both the assays were used to measure the total anti-oxidant power of the hot water plant extracts by "in vitro" reactions. The Total Anti-oxidant Capacity (TAC) assay is based on a redox reaction between a test compound and copper II, Cu (II). If a test compound has got reducing power, the Cu (II) is readily converted into Cu (I) which can be monitored by the addition of a chromophore termed Bathocuproine (BATO). Fifty µl of hot water plant extracts solution in PBS (at concentrations ranging from 0.031 mg.mL-1 to 4 mg.mL^{-1} to R. frangula and C. myrrha) was aliquoted into a 96-well plate. 50µl of a BATO solution (360µM) was added to each well and the background absorbance at 490nm was measured, then 25µl of 100µM CuSO4 solution was added to each sample and incubated at room temperature for 30 min. At the end of the incubation time, the absorbance at 490 nm was measured by a plate reader (Victor3, PerkinElmer). As reference standard, scalar dilutions of CuCl were used, ranging from 30 to 10 mM.

Oxygen Radical Absorbance Capacity (ORAC) assay is based on the ability of a test compound to inhibit the oxidation of a fluorophore, generally fluorescein, by a potent oxidant, 2,2_- azobis (2-amidinopropane) dihydrochloride (AAPH). Twenty-five µl of hot water plant extracts dilutions in phosphate buffer 75mM, pH 7.4 (at concentrations ranging from 0.031 mg.mL^{-1} to 4 mg.mL^{-1} to *R. frangula* and *C. myrrha*) was aliquoted into 96-well plate and 150µl of fluorescein solution (2.5 nM in phosphate buffer) was added to each sample. After incubation at 37 °C for 15 min, 25µl of AAPH solution (153mM in phosphate buffer) was pipetted into each well and the progress of the reaction monitored at 535 nm, using a fluorescence multi-well reader. The fluorescence was measured every minute for 5 min.

Antioxidant capacity of plant extracts on HaCaT cells

2 10^4 HaCaT cells are maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS under 5% CO_2 at 37°C, were seeded in 96-well plates and grown for 20 h to let them attach to the bottom of the well. The cells were then incubated with different concentrations of hot water plant extracts or ascorbate 250µM, used as positive control, for 2 h. At the end, the cells were washed in PBS and the cell auto-fluorescence value (background) measured at the plate reader, using 490nm as excitation and 535nm as emission wavelength. The cells were then incu-

bated with the dye CM-H_2DCFDA (5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, Invitrogen) at 37°C for 30 min. After an additional wash in PBS, the cells were treated with H_2O_2 150µM and incubated for 30 min. The fluorescence of the samples was thus measured at 535nm (excitation 490 nm), using the instrument (Flexstation 3, PRIMACEN), and from each measure the cell auto-fluorescence value was subtracted.

RNA extraction and reverse transcription

Total RNA was extracted using an RNeasy mini kit (Qiagen) in accordance with the manufacturer's instructions. The RNA was eluted with 22 µl of RNase-free water. DNase I treatment (RNase-Free DNase Set; Qiagen) was performed to remove contaminating genomic DNA. The quality of the RNA was assessed, and the quantity was estimated by nanodrop.

Approximately 1µg of RNA were reverse-transcribed in a 20 µl reaction by High Capacity cDNA Reverse Transcription Kits (Applied biosystem) that contained 10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix (100 mM), MultiScribe™ Reverse Transcriptase 50 U/µL and RNase Inhibitor.

Quantitative real-time PCR amplification reactions

Quantitative real-time PCR amplification reactions were performed using a 7500 Fast Realtime PCR System (Applied Biosystems) in a final reaction volume of 13µl, which contained fast SYBR Green Master Mix (Applied Biosystems), 300 nM forward and reverse primers and the template cDNA. The thermal cycling conditions involved a denaturation step at 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The final dissociation step was performed at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s.

Results

ROS production by HaCaT cells in response to plant extracts

To determine if treatment with plant extracts induced oxidative stress in keratenocytes HaCaT, ROS production was monitored based on different concentrations with the probe CM-H_2DCFDA able to detect all the ROS.

The hot water extract of *R. frangula* generates ROS in HaCaT cells. Moreover, production of ROS by HaCaT cells increases with increasing concentrations (figure 1). Indeed, after 60 minutes of treatment with *R. frangula* hot water extracts, a significant increase of ROS production is observed for the following concentrations 0.5 mg.mL^{-1}, 1 mg.mL^{-1}, 2 mg.mL^{-1}, 3 mg.mL^{-1} et 4 mg.mL^{-1} compared with HaCaT cells treated with PBS (Phosphate Buffer saline) buffer only (figure 2). After two hours of treatment, the generation of ROS continues to increase to reach an equivalent fluorescence 200 AU, and 2 times higher than the PBS control (figure 2).

The hot water extract of *C. myrrha* gererate ROS in HaCaT cells within the first hour after treatment (figure 1). Moreover, the concentration increases and more production of ROS by HaCaT cells is observed. After 60 minutes of treatment with *C. myrrha* hot water extracts, a significant increase of ROS production is observed for the concentrations 0.5 mg.mL^{-1}, 1 mg.mL^{-1}, 2 mg.mL^{-1}, 3 mg.mL^{-1} et 4 mg.mL^{-1} compared to HaCaT cells treated with PBS (figure 2). However, after sixty minutes, the trend reverses. The hot water extract of *C. myrrha* does not induce the generation of ROS compared to HaCaT cells treated with the buffer PBS. After 120 min with the treatment PBS, the HaCaT cells produce much more ROS than HaCaT cells treated with the hot water extract of *C. myrrha* (figure 2).

Figure 1. Production of reactive oxygen species (ROS) in HaCat cells treated with at *Commiphora myrrha* and *Rhamnus frangula* extract. After tratement the amount of ROS produced in the cells measured by the fluroscent probe CM-H_2DCFDA. The values are means of 3 independent measures obtained from one reprasentative experiment.

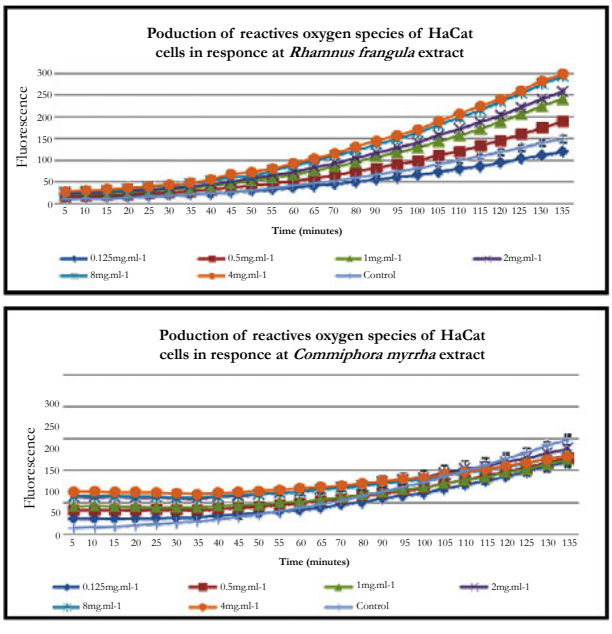
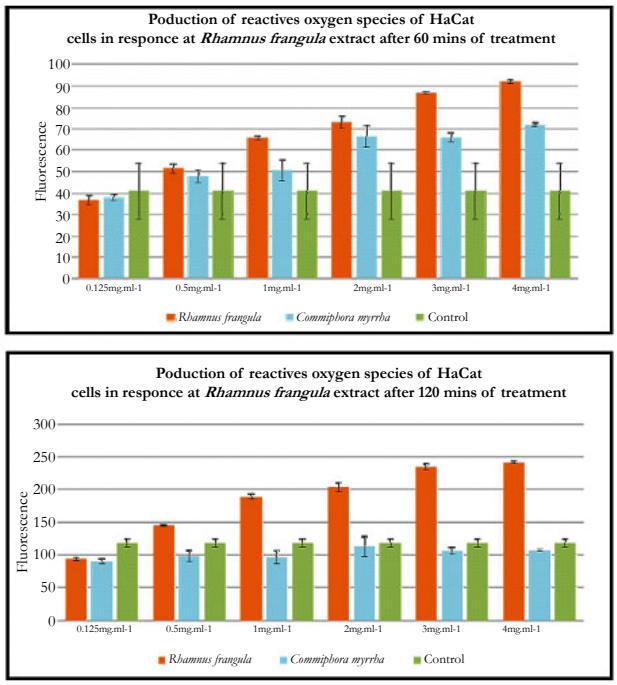


Figure 2. Production of reactive oxygen species (ROS) in HaCat cells treated with at *Commiphora myrrha* and *Rhamnus frangula* extract at 60 and 120 mins. After tratement the amount of ROS produced in the cells measured by the fluroscent probe CM-H_2DCFDA. The values are means of 3 independent measures obtained from one reprasentative experiment.



Viability and proliferation of HaCaT cells in response to plant extracts

In order to determine if the hot water of different plant extracts are cytotoxic to HaCaT cells, the cell viability and cell proliferation were determined by the MTT assay at different concentrations according to the method of Apone et al., [1].

Treatment with the hot water extract of *R. frangula* reduces the viability of the cells and proliferation at concentrations higher than 0.5 mg.mL^{-1}. However, concentrations below 0.5 mg.mL^{-1} had no significant effect on the viability and proliferation of HaCaT cells. Treatment with the hot water extract of *C. myrrha* reduces the viability and proliferation at concentrations higher than 1 mg.mL^{-1}. However, concentrations below 1 mg.mL^{-1} have no significant effect on the viability and proliferation of HaCaT cells (figure 3).

Anti-oxydant activity of plant extracts

***In vitro* assays**: The anti-oxidant activity of a compound or a mixture of compounds depends on its total reducing capacity, on its ability to penetrate into the cells, and on its capacity to trigger a signal cascade leading to the production of endogenous anti-oxidant compounds in the cells. "*In vitro*" assays measure the reducing potential of the test material; however, they do not provide any information on the anti-oxidant induction in living cells.

Total Anti-oxidant Capacity (TAC): The Total Anti-oxidant Capacity (TAC) assay is based on a redox reaction between a test compound and copper II, Cu (II). If a test compound has a reducing power, the Cu (II) is readily converted into Cu (I) which can be monitored by the addition of a chromophore termed Bathocuproine (BATO).

For the hot water extracts of *R. frangula* and *C. myrrha* different concentrations (ranging from 0.062 mg.mL-1 to 4 mg.mL-1) were mixed with 100mM of Cu (II) and BATO, and after 30 min the amount of Cu (I) produced was measured at 490 nm.

For the concentrations ranging from 0.062 mg.mL^{-1} to 0.5 mg.mL^{-1} of the hot water extract of *R. frangula*, the quantity of mmol of Cu (I) produced per liter remains constant and is equivalent to 25 mmol. However, with increasing concentrations of *R. frangula* hot water extracts, the quantity of Cu (I) product per liter increases to reach 45 mmol at a concentration of 4 mg.ml^{-1}. In the presence of the PBS (control), the amount of mmol of Cu (I) produced is equivalent to zero.

For the concentrations, ranging from 0.062 mg.mL^{-1} to 4 mg.mL^{-1} of the hot water extract of *C. myrrha*, the quantity of mmol of Cu (I) produced per liter increased in relation with the concentrations used to reach 42 mmol at a concentration of 4 mg.ml^{-1}. In the presence of the PBS (control), the amount of mmol of Cu (I) produced is equivalent to zero (figure 4).

Oxygen Radical Absorbance Capacity (ORAC): Oxygen Radical Absorbance Capacity (ORAC) assay is based on the ability of a test compound to inhibit the oxidation of a fluorophore, generally fluorescein, by a potent oxidant, 2, 2- azobis (2-amidinopropane) dihydrochloride (AAPH). The standard is a powerfull anti-oxidant, the trolox (vitamin E) with the concentration ranging from 0.5 µM to 20 µM.

For the hot water extract of *R. frangula* at the concentration of 0.031 mg.mL^{-1}, the values are comprised between the curves of the trolox equivalent to 5 µM and 8 µM. Thus, the antioxidant activity of hot water extracts of *R. frangula* is equivalent to the concentration of trolox 5 - 8µM. At the concentration 0.062 mg.mL^{-1} the curve is located after the curves of the trolox equiva-

Figure 3. Cytotoxic effects of *Rhamnus frangula* and *Commiphora myrrha* extract extracts on HaCaT cells. Cells were teated with the exctacts for 72 h. Cell viability was measured by the MTT assay. Data are averages of triplicate determinations.

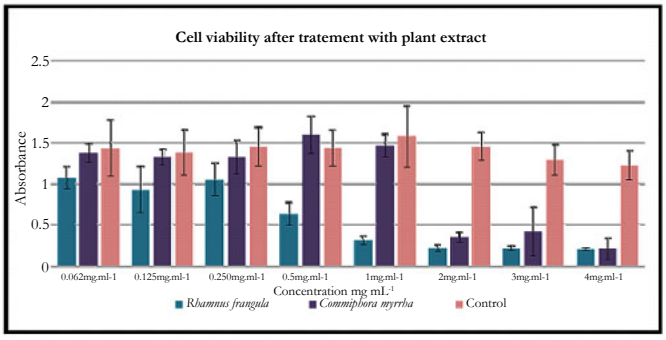
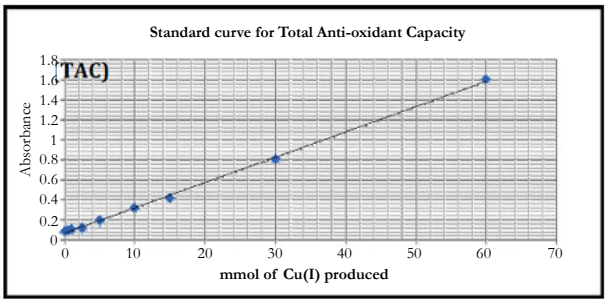
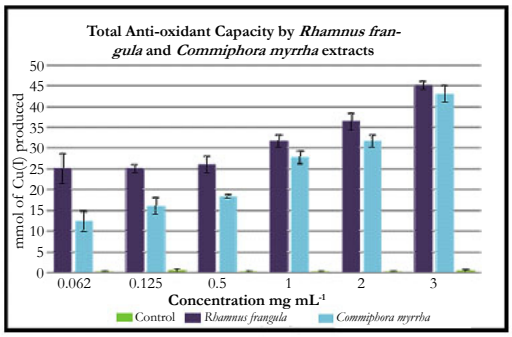


Figure 4. Standard curve for total anti-Oxidant Capacity (b) Total anti-oxidant power of *Rhamnus frangula* and *Commiphora myrrha* extract measured by Total Anti-oxidant Capacity (TAC) assays. Six different caocentrations of the plant extract were analyzed for their anti-oxidant power by using the Total Anti-oxidant Capacity (TAC) assay. The values reported exported in the graphs are avarages of three independent measures and the error bars represent standard deviations.





lent to 10µM. Thus, the antioxidant power of hot water extract of *R. frangula* is equivalent to the concentration of trolox higher at 10µM of trolox (figure 5).

For the hot water extract of *C. myrrha* at the concentration of 0.125 mg.mL^{-1} and 0.250 mg.mL^{-1}, the curve is located between the curves of the trolox equivalent to 0.5 µM and 5 µM. Thus, the antioxidant power of hot water extract of *R. frangula* is equivalent to the concentration of trolox 0.5-5 µM (figure 5).

2 In vivo assays: To evaluate the protective anti-oxidant effect of the plant extract on the HaCaT cells, we treated the cells with eight different concentrations of the mixture before being stressed by H_2O_2.

For the hot water extract of *R. frangula* ranging from 4 mg.mL^{-1} to 0.250 mg.mL^{-1}, a strong reduction of ROS production by HaCaT cells was observed compared to the untreated sample. For the concentration ranging from 0.125 mg.mL^{-1} to 0.062 mg.mL^{-1} a little reduction of ROS production was observed compared to the untreated sample (figure 6). Indeed, after 60 minutes of treatment, a strong reduction in the production of ROS is observed for concentrations ranging from 4 to 0.250 mg .ml-1 (figure 7). However, after 120 minutes of treatment a sharp decline in the production of ROS is observed for concentrations ranging from 4 to 1 mg.mL^{-1} (figure 7). It could be that the antioxidant capacity of the *R. frangula* hot water extract has been consumed at the level of the low concentrations.

For the hot water extract of *C. myrrha*, a strong reduction of ROS production was observed compared to the untreated sample only for the concentration at 4 mg.ml^{-1}. For the other concentration (3 to 0.062 mg.mL^{-1}), there is no reduction of ROS production by HaCaT cells (figure 6). Indeed, after 60 minutes and 120 minutes, a decrease in the production of ROS is observed only for the highest concentration (4 mg.mL^{-1}) (figure 7).

Microbial activity of plant extracts

The bright field microscopy of skin microbial flora shows the presence of bacteria in cluster or in diplococcic (figure 8). These morphologies of bacteria suggest they belong to the genus Staph-

Figure 5. Total anti-oxidant power of *Rhamnus frangula* and *Commiphora myrrha* extract measured by Oxygen Radical Absorbance Capacity (ORAC) assays. Six different caocentrations of the plant extract were analyzed for their anti-oxidant power by using the ORAC assay. The values reported exported in the graphs are avarages of three independent measures. The arrows represent the samples.

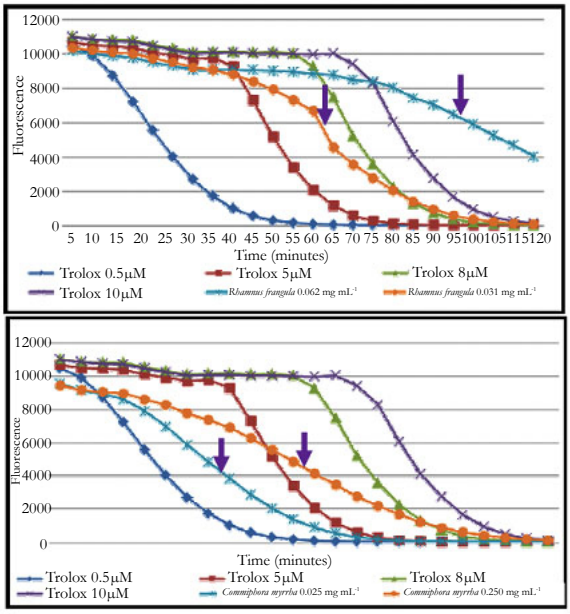
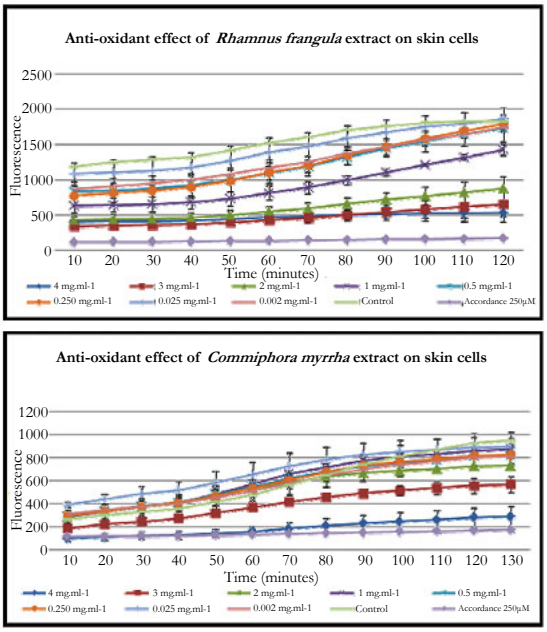


Figure 6. Anti-oxidant effect of *Rhamnus frangula* and *Commiphora myrrha* extract against H_2O_2 on HaCat cells.



ylococcus and Streptococcus.

The microbial activity of the plant extracts of *R. frangula* and *C. myrrha* were studied *in vitro* with different concentrations (0.25, 0.5, 1, 2, and 3 mg/ml) against skin microbial flora.

The enumeration of bacteria shows a strong increase in proliferation of skin microbial flora treated with 0.25 $mg.ml^{-1}$ hot water extract of *R. frangula* compared to untreated samples (figure 9). For the concentration ranging from 0.5 $mg.mL^{-1}$ to 3 $mg.mL^{-1}$, the concentration of bacteria is equivalent to that of untreated samples.

The enumeration of bacteria shows an increase in skin microbial flora growth treated with 0.25 $mg.ml^{-1}$ hot water extract of *C. myrrha* compared to untreated samples (figure 9). For the concentration ranging from 0.5 $mg.mL^{-1}$ to 3 $mg.mL^{-1}$, the concentration of bacteria is equivalent to that of the samples treated with 0.25 $mg.ml^{-1}$ of hot water extract of *C. myrrha*. The treatment with 1 ml of ethanol is the negative control.

Effects of plant extract on defense gene activation in HaCaT cells

In order to investigate whether hot plant extract activate defense gene markers in HaCaT cells, we examined the expression changes of some selected genes in response to extracts from *R. frangula* and *C. myrrha* at 0.25 $mg.ml^{-1}$ over a time course by real-time

Figure 7. Anti-oxidant effect of *Rhamnus frangula* and *Commiphora myrrha* extract against H_2O_2 on HaCat cells after 60 and 120 minutes of treatment.

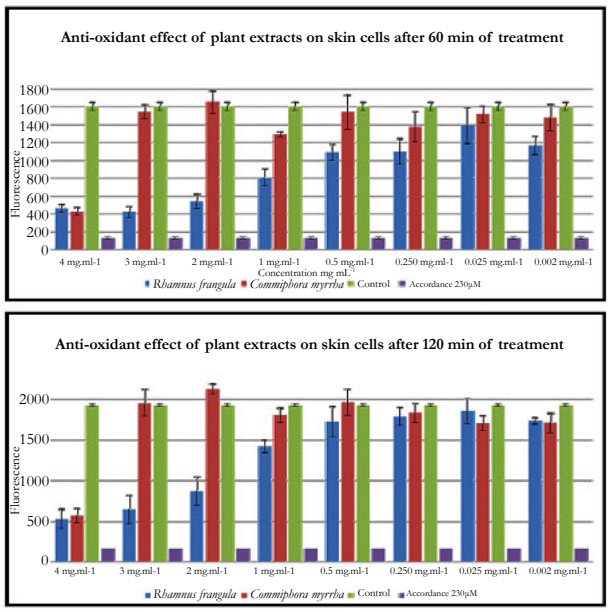


Figure 8. Bright field microscopy of skin microbial flora.

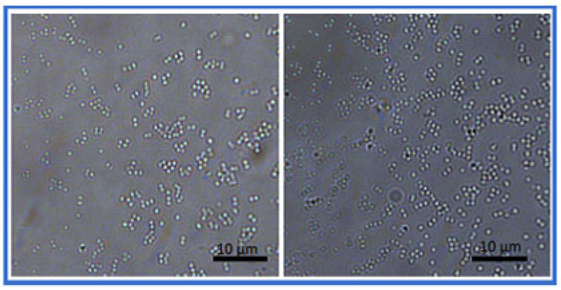
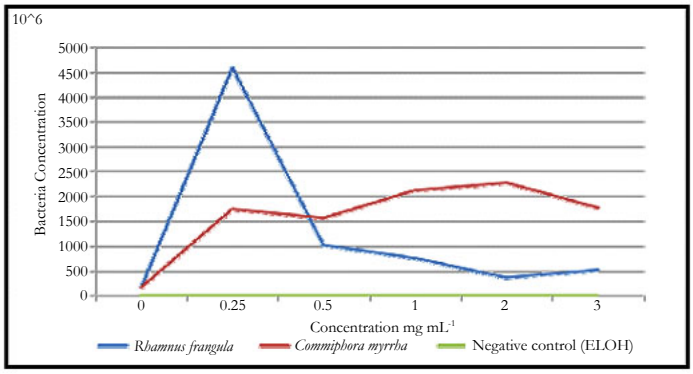


Figure 9. The enumeration in in vivo of skin microbial flora treated with *Rhamnus frangula* and *Commiphora myrrha* extract. After treatment, the concentration ofskin microbial flora increase.



quantitative reverse transcription (qRT)-PCR (figure 10). We have chosen this concentration because it is a non-lethal concentration to the cells.

For this study we have selected five genes: CATALASE (CAT), SUPEROXIDE DISMUTASE (SOD), KERATIN1 (KER1), DEFENSIN1 (DEF1), INTERLEUKIN (S100A). The gene CAT encoding a catalase that is important for protecting the cells from oxidative damage caused by the ROS. After treatment with the extracts from both *R. frangula* and *C. myrrha*, the expression of CAT gene is not induced (figure 11 and 12). The SOD gene encodes for an enzyme that catalyses the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen. After treatment with hot plant extract of *R. frangula*, we observe a reduction in SOD transcript at 1, 3, 6, 12 h after elicitation (figure 11). After treatment with hot plant extract of *C. myrrhaan* increase in SOD transcript at 1 h after elicitation (figure 12). The KER1 gene is a differentiation factor. The protein encoded by this gene is a member of the keratin gene family. The type II cytokeratins consist of basic or neutral proteins that are arranged in pairs of heterotypic keratin chains coexpressed during differentiation of simple and stratified epithelial tissues [5, 8]. This gene is down regulated at 24, 48 h after treatment with hot plant extract of *R. frangul* and *C. myrrha*. An induction of DEFENSINE 1 gene, one of the most important genes known to encode for a peptide with

Figure 10. Procedure to ectraction of RNA in HaCat cells after treatment with hot water extract of *Rhamnus frangula* and *Commiphora myrrha* at 1h, 3h, 6h, 12h and 48h compared at HaCat cells untreated.

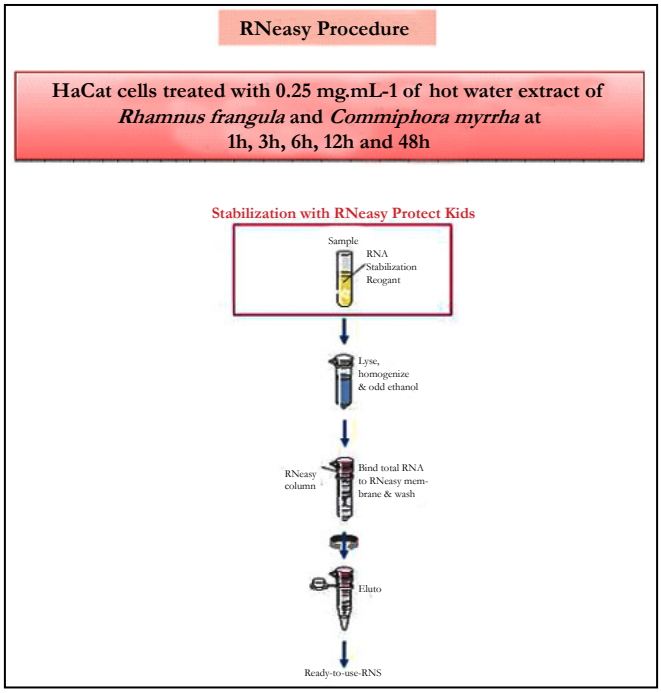
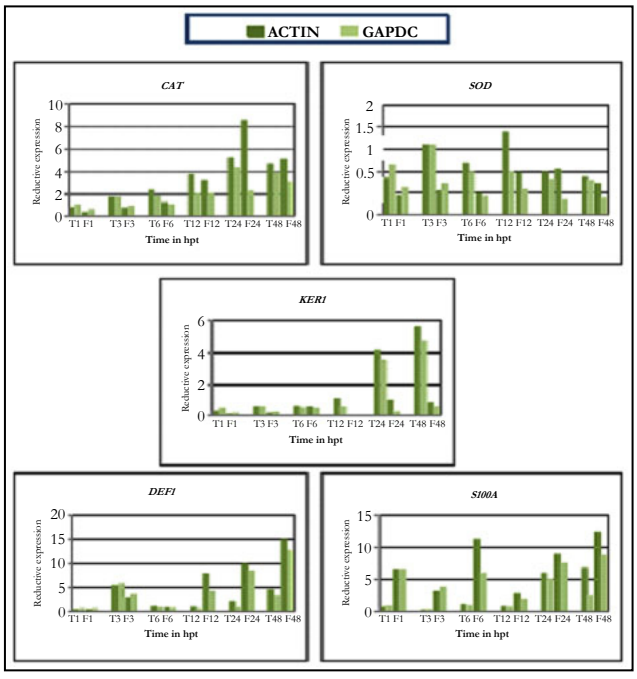


Figure 11. Relative expression levels of *CAT, SOD, KER1, DEF1 and S100A* in HaCat cells in responce to hot water extract of *Rhamnus frangula*. hpt, Hours post treatment, Gene expression values are presented as relative expression. The reference genes used in this study are *ACTIN* and *GAPDC*.

CAT: CATALASE, DEF: DEFENSINE1, hours post treatment, KER1: KERATINE, S100A: INTERLEUKIN, SOD: SUPEROXIDEDISMUTASE

T: untreated

T: treated with the extract.

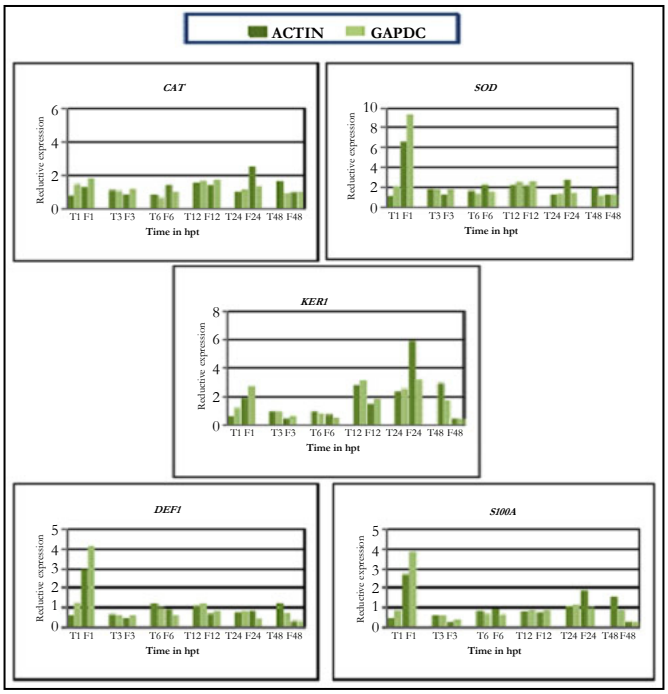


a strong antimicrobial activity occurs. This human defensin is up-regulated from 12, 24, 48 h after elicitation with hot plant extract of *R. frangula* treatment (figure 11) and is up-regulated from 1 h with hot plant extract of *C. myrrha* (figure 12). The gene S100A gene encoding an interleukin that participate in the regulation of immune responses, inflammatory reactions. After treatment with hot plant extract of R. frangula, we observed an up-regulation at 1, 3, 6, 12, 24, 48 h after treatment (figure 11). After treatment with hot plant extract of *C. myrrha* an increase in S100A transcript at 1 h after elicitation (figure 12).

Discussion

The protective effects of extracts from both plants (*R. frangula and C. myrrha*) endemic to Saudi Arabia were assessed on HaCaT cells. ROS production is a well known marker of stress response.

Figure 12. Relative expression levels of *CAT, SOD, KER1, DEF1 and S100A* in HaCat cells in responce to hot water extract of *C. myrrha*. hpt, Hours post treatment, Gene expression values are presented as relative expression. The reference genes used in this study are *ACTIN* and *GAPDC*.
CAT: CATALASE, DEF: DEFENSINE1, hours post treatment, KER1: KERATINE, S100A: INTERLEUKIN, SOD: SUPEROXIDEDISMUTASE
T: untreated
T: treated with the extract.



In order to determine whether plant extracts are able to trigger an immune response in HaCaT cells, ROS formation was monitored using the fluorescent probe CM-H_2DCFDA.

Extracts of both plants triggered ROS formation in HaCaT cells. The hot water extracts from *R. frangula and C. myrrha* gererate ROS in HaCaT cells within the first hour after treatment. However, the intensity of the response was more important regarding the hot water extract from *R. frangula* as compared to *C. myrrha*. These findings reveal that extracts from these two saoudian plants are able to stimulate an immune response in HaCaT cells.

The next step was to determine the range of concentration the most appropriate for using these products as elicitors. In order to determine if the plants extracts are cytotoxic to HaCaT cells, cell viability and proliferation were determined by the MTT assay, according to the method of Apone et al[1, 8]. For the R. frangula extract, a loss of cell viability and proliferation occurred at concentrations higher than 0.5 mg.mL^{-1}. The *C. myrrha* extract reduces the viability and proliferation at concentrations higher than 1 mg.mL^{-1}. As a consequence, these products should not be used at such higher concentrations. We consider that these extracts from *R. frangula* and *C. myrrha* are not suitable for use as potential elicitors. Indeed the concentration required to induce ROS is higher than the toxicity value.

However, to rule out the possibility that the fluorescent probe CM-H_2DCFDA is not sufficiently sensitive in the cell system investigated, expression of defense gene markers was investigated in HaCaT cells in response to 0.25 mg.ml^{-1} treatment of extract from *R. frangula* and *C. myrrha*. The expression of selected marker genes was monitored in response to extracts from *R. frangula* and *C. myrrha* at 0.25 mg.ml^{-1} over a time course by real-time quantitative reverse transcription (qRT)-PCR. Surprisingly, the extracts from both *R. frangula* and *C. myrrha* induce expression of defense genes in HaCaT cell at such concentration. Moreover, the extract from *R. frangula* induces the expression of more strongly defence gene than extract from *C. myrrha*. These data confirmed that the fluorescent probe CM-H_2DCFDA was not sensitive enough to detect ROS production in response to low concentration of plant extracts.

As a consequence, based on (qRT)-PCR data, extracts from *R. frangula and C. myrrha* could be potential elicitors to trigger immune response in HaCaT cells.

We investigated the potential property of these fractions to be used as anti-oxidant molecules. To determine the anti-oxidant capacity of the hot water extract of *R. frangula and C.myrrha* we performed the two following in vitro tests: The Total Anti-oxidant Capacity (TAC) and Oxygen Radical Absorbance Capacity (ORAC). Additionally, an *in vivo* assay was performed. Our findings reveal that the different plant extracts have a strong in vitro anti-oxidant capacity. For the anti-oxidant effect of *R. frangula and C. myrrha* H_2O_2 on HaCaT cells both plant extracts protect the cell against the adverse effects of H_2O_2. Interestingly, our findings reveal that both plant extracts could be used as potential anti-oxidant molecules against oxidative stress. It should be noted that concentrations used are very low and below the limit value for toxicity.

To evaluate the effect of the hot water extract of *R. frangula and*

C. myrrha skin microbial flora, we have performed one test *in vitro*. The microbial activity of both plant extracts were studied *in vitro* at different concentrations (0.25, 0.5, 1, 2, and 3 mg/ml) against skin microbial flora. The enumeration of bacteria shows a strong increase in skin microbial flora growth treated with 0.25 $mg.ml^{-1}$ hot water extract of *R. frangul*a compared to the non-treated flora. The enumeration of bacteria shown an increase in skin microbial flora growth treated with 0.25 $mg.ml^{-1}$ hot water extract of *C. myrrha* compared to the skin microbial flora untreated. Thus, the different plant extracts used here are not harmful to the natural cutaneous microbial flora.

The results obtained with extracts from two endemic plants of Saudi Arabia *R. frangula and C. myrrha* are highly promising with regards to skin protection.

The limit value for toxicity of the extracts from *R. frangula and C. myrrha* is 0.5 mg.mL-1 and 1 mg.mL-1 respectively.

Both extracts have a significant anti-oxidant activity. Therefore these extracts could be used as potential anti-oxidant molecules against oxidative stress. It is worth noting that concentrations used are below the limit value for toxicity.

The extracts have no negative effect for the natural cutaneous microflora.

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