Effects of Activated Kaolin, Quicklime and Paraffin on Sunburn, Fruit Surface Temperature, Antioxidant Activity, Total Polyphenols and Anthocyanin's of Saveh Pomegranate Fruit

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Received: 23 Nov 2020; Accepted: 18 Dec 2020

Abstract
Pomegranate is one of the most important strategic crops in Saveh city, Markazi province, Iran. The cultivation area of Saveh pomegranate orchards is about 10 thousand hectares and 15 tons of pomegranates were harvested per hectare. One of the problems faced by pomegranate fruit cultivation is suffering from cracking and sunburn during summer season. Control measures done include the use of pesticides and/or integrated management practices for the management of these problems, but these control methods are either expensive, non-environmental-friendly, or lack efficacy. Therefore, in this study we investigate the effect of three types of sunscreen whereby a field experiment was performed on Saveh pomegranate fruit in a randomized complete block design with 4 treatments and 3 replications. Mulberry pomegranate fruits were treated with three sunscreens including: activated kaolin (60 gL^{-1}), quicklime (40 mlL^{-1}), liquid paraffin (40 ml/fruit) and control treatment (without any material) over a period of 4 months. The results showed that all treatments caused a reduction in fruit sunburn. The amount of polyphenols in pomegranate juice increased significantly only in the treatment with liquid paraffin. The antioxidant and anthocyanin levels of pomegranate peel increased significantly compared to the control under all three treatments. This study suggested that active kaolin as an effective sunscreen compound with significant increase in anthocyanin, antioxidant and total phenol of the peel skin.

Keywords: Pomegranate Fruit, Sunscreen Compounds, Fruit Surface Temperature, Polyphenols, Antioxidants, Anthocyanin

Introduction
Pomegranates are an ancient crop in Iran and its production and harvested area are more than 700,000 tons per year and 56,000 hectares, respectively. The new industry will provide both fresh fruit and fruit processed for juice. In this work the pomegranates were grown in Saveh, 120 km south of Tehran (Capital of Iran) which is located in Markazi province where summer temperatures can be over 40°C for several days at a time. These high temperatures can cause sunburn damage to the outside skin of the fruit making then unsaleable. Brito *et al.,* (2019) have shown that the use of kaolin sunscreen treatments can significantly reduce sunburn damage. Similar results have been reported for apples

(Glenn, 2016). EL-Gioushy *et al.,* (2017) reported, the increase in most fruit quality measurements over control was significantly with 3% $CaCO_3$ sprayed of Keitt mango trees. Kumar & Singh (2018) had similar results about using liquid paraffin on mango fruits. Our work looked at the effect of applying sunscreen treatments to pomegranate fruit on fruit temperature as the index of sunburn damage. We also looked at the effect of sunscreens on the internal antioxidant concentration of the juice. Both factors are important in terms of fruit quality.

This high temperature caused stress in plants, which leads to the production of reactive oxygen species, resulted in oxidative stress (Ma *et al.*, 2008). Plants protect themselves from the cytotoxic effects of the active oxygen species by antioxidant enzymes or metabolites such as glutathione, ascorbic acid and carotenoids which may scavenge reactive oxygen (Ma *et al.*, 2008). In pomegranate fruit protective antioxidant metabolites include polyphenols such as ellagitannins, tannins, anthocyanins and flavonoids (Mousavinejad *et al*., 2009). The level of the antioxidant metabolites in plants changes in response to abiotic stress. For example, heating apple leaves from 28 to 40°C increased the content of total ascorbate, ascorbic acid, total glutathione, and glutathione initially but after a high temperature exposure of more than 2 hours the contents of these antioxidant compounds declined (Ma *et al.,* 2008). Furthermore, in sunburnt apple peel an up-regulation of the antioxidant system in response to the increased reactive oxygen species generated by the high light and high temperature exposure was shown (Chen *et al*., 2008). However, the researchers concluded that the up-regulation in response to photo oxidative stress was not enough to protect against the photo oxidative damage and hence the resulting visual sunburn damage.

The aim of this work was to evaluate the effectiveness of three commercial sunscreen treatments activated kaolin, quicklime, and liquid paraffin for preventing sunburn damage of pomegranate fruit grown in Saveh, Markazi province, Iran. The experiment also looked at the effect of external sunburn on the, growth, antioxidant and anthocyanin contents and total phenols of the fruit skin and fruit juice.

Methodology

The experiments were performed in a completely randomized block design with three replications in a private garden in the gardens of Seyed Gholi region, 12 km of Saveh-Hamadan road in Markazi province in Iran during spring of 2017. In mid-June, 9 pomegranate trees of Saveh Malas variety were randomly selected. Three sunscreens including activated kaolin (60 gL^{-1}), quicklime (40 mlL^{-1}) and liquid paraffin (40 ml/fruit) liquid paraffin (Bemo), 85% product in Iran. One month after fruit formation, the fruits are coated and this process is repeated every week, in addition to the treatment, there is also a control treatment. Fruits were counted in terms of sunburn and their average is expressed as a percentage in the results. Surface temperature of treated and control fruits were measured too. Immediately after fruits harvesting, the skin was separated from the seeds and stored in a freezer at -18 °C prior to biochemical tests.

Measurement of surface temperature of pomegranate fruits

Surface temperature of fruits and surrounding environment was measured three times, in the morning (9:30), noon (12:30) and evening (16:30) in the middle of July 2015 by using mercury thermometer so that each time the temperature of the upper, middle and lower fruit surface in the bush was measured and then statistical analysis was performed and the average ambient temperature was 40.6°C.

Relative growth rate of pomegranate fruits

The relative growth rate (RGR), called the efficiency index by Black man, shows growth as the rate of increase in size per unit of size. This parameter allows a fairer comparison with the absolute growth rate. RGR is usually measured in terms of dry weight, and its literal definition can be attributed to the rate of production of organic compounds obtained in the plant. Negative RGR values indicate the loss of organic compounds in fruits (Hunt, 1997).

The relative growth rate can be obtained from the following equation:

$$RGR = \frac{L_n W_2 - L_n W_1}{t_2 - t_1}$$

L_n: Natural logarithm
W_1: total plant dry weight at time t_1 in grams
W_2: dry weight of the whole plant at time t_2 in grams
$t_2 - t_1$: Growth time during treatment

The RGR unit is: grams of dry matter produced per gram of plant per day
RGR = $gg^{-1}d^{-1}$

Preparation of fruits to measure secondary metabolites.

To measure the amount of chemical compounds in the skin and pomegranate juice, fruits were taken out of the freezer one by one from all treatment. For each treatment, 9 trees and from each tree 20 fruits that were at a relatively equal height from the ground were selected and treated. To prepare pomegranate juice, first the seeds are separated from other parts of the fruit by squeezing. The juice and the fruit peel (dried in an oven at 60°C for 24 hours) were extracted by using alcohol (methanol) and centrifuged (Hetticch) at 3000 rpm for 10 minutes. The supernatant was isolated and used for further testing. The method used was according to Chanwitheesuk *et al.,* (2005), with minor modifications.

Measurement of antioxidant activity of pomegranate peel and juice by DPPH method.
The method used for measurement of DPPH activity was based on Tang *et al.*, (2008). DPPH radicals are more stable than hydroxyl radicals and superoxide anions, and this is one of its advantages, and phenolic compounds and other known antioxidants can neutralize this radical. Pomegranate fruit due to having large amounts of phenolic compounds and a high level of total antioxidant capacity has shown great power in neutralizing DPPH radicals (Nabavi, 2009). The stable radical diphenyl-picrylhydrazyl was used to determine the activity of free radical trapping. At this stage, 4 mg of the radical stable pheny phenyl picryl hydrazyl or DPPH (black) from Sigma-Aldrich with code D9132 dissolved in 100 ml of methanol. In this case, a solution with a concentration of 0.04 mg/ml was obtained, then 2 ml of DPPH was poured into all the tubes with the addition of 2 ml of skin extract. Add pomegranate fruit or fruit juice to DPPH. Afterwards, the tubes are placed in a dark environment for 15 minutes and read at a wavelength of 517 nm by using Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The experiments are repeated three times to obtain average reading. The tube that has the most extract will be yellow and the lowest concentration is purple, which related to DPPH. The numbers obtained by the following formula are converted to the percentage of inhibition: Subtract the sample absorption number from the control number and divide by the control, so we have one percent inhibition for each absorption. A calibration curve is drawn using the vitamin C standard. The equation obtained is similar to the equation:

$$y = 56.73x+17.03.$$

Here y is the same percentage of inhibition as x is obtained as the antioxidant content in milligrams of vitamin C per gram of dry extract (fruit peel) or milliliters of juice.

Ac: Control absorption
As: Sample absorption
% I: Free Radical Inhibition (DPPH)

Measurement of total skin phenol and juice
The method was adopted based on Goosen *et al.* (2018). After taking 0.5 ml of methanolic extract (25% water + 75% methanol), mix the skin or fruit juice with 5 ml of Folin–Ciocalteu reagent (1 to 10 diluted with distilled water), and then add 4 ml sodium carbonate (1 M) (10.6 g per 100 ml of distilled water). For the control, distilled water is poured instead of the extract, and then Folin–Ciocalteu reagent and sodium carbonate was added. This solution was used for calibration of the spectrophotometer. The above solution is placed in the dark for 15 minutes and then read the absorbance at a wavelength of 765 nm. The calibration curve is plotted using the gallic acid standard from Sigma-Aldrich. The equation obtained is similar to the equation: $y = 0.008x + 0.032$. Here y is the number read in front of the control. X is obtained as the phenolic content in milligrams of gallic acid per gram of dry extract (fruit peel) or milliliters of juice. The experiments are repeated three times to obtain the average.

Investigation of the activity of anthocyanin compounds
Determination of activity of anthocyanin compounds was done using method by Coklar & Akbulut (2017). 0.5 g (pomegranate seed or dry pomegranate peel powder) is ground in a solution containing 99 ml of methanol and 1 ml of hydrochloric acid, then placed in the refrigerator for 24 hours. After a certain time, the solution is centrifuged, and it is poured into a Unico (2150) spectrophotometer tube and the light reflection is read in 2 wavelengths and is calculated according to the following formula:

(Absorbance in 675nm×1.4) - (Absorbance in 530nm) + Anthocyanin content

The amount of anthocyanin was in milligrams per 0.5 grams of fruit. All samples are repeated three times and then averaged.

Statistical analysis
The results were expressed as the mean ± standard Error (SE) and subjected to one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. The statistical analyses were performed using MINITAB (Release 14, Minitab Inc. USA).

Results & Discussion

In this study, treatments of fruits with activated kaolin, quicklime and liquid paraffin have successfully been done (Figure 1). From the results in Table 1, it shows that the use of sunscreen has reduced the surface temperature of the fruit, which is consistent with the results of Sharma *et al*., (2020). From the findings of this study, it showed that a decrease in fruit surface temperature was observed in all three treatments, with kaolin caused the lowest decrease in fruit surface temperature compared to the control (Table 1). It was observed that there were no differences between relative growth rates of pomegranate fruit on the different treatment of sunscreens. Although the temperature of the fruits decreases of 2 to 3 degrees in the application of sunscreens, but the differences were not statistically significant.

Total phenolic content, DPPH radical and anthocyanin values were considerably good for pomegranate whether in fruit extracts or skins. These parameters were directly correlated with the use of sunscreens. From this study, the highest significant amount of phenol compounds was observed in fruit juice with liquid paraffin treatment and in fruit skin under active kaolin treatment. The amount of total polyphenols in pomegranate juice treated with paraffin showed a significant increased at the level of 5% probability compared to control.

We also find that the use of sunscreen treatments with an effect on lowering the temperature increases antioxidants content. The increase of antioxidants contents in fruit juice is mostly related to paraffin and quicklime treatments. Whereas in the skin extract of the fruit, it is more concerned with the treatments of kaolin and quicklime. This could be due to the reducing of temperature, which related to escalating of antioxidant compounds. Complete antioxidant activity is also attributed to the levels of phenolic acids, ascorbic acid, and anthocyanins (Kulkarni & Aradhya, 2005; Sharma *et al.,* 2020). Pomegranate fruit contains protective antioxidant metabolites, including polyphenols such as alginates, tannins, anthocyanins, and flavonoids (Mousavinejad *et al.,* 2009). As antioxidants increase, polyphenols concentration also increases, as evidenced in our research.

As for anthocyanin and antioxidant activity in the fruit juice, there are no significant increased for all treatments. For fruit skin, treatment with activated kaolin showed significant increase for all anthocyanin, antioxidant activity and total phenol (Table 1). It is also exhibited that antioxidant activity increased significantly in all the treated fruit skin. The stability of anthocyanins changes with temperature, pH, light, and oxygen and is sensitive to degradation by oxidizing enzymes (Alighourchi *et al.,* 2007; Jaiswal *et al.,* 2009). Anthocyanins are polyhydroxy glucosides and polymethoxy derivatives of the flavilium cation (Kong *et al.,* 2003), which is responsible for the red color of pomegranate fruit.

A: activated kaolin (60 gL^{-1})

B: quicklime (40 mlL^{-1})

C: liquid paraffin (40 ml/fruit)

Figure 1: Treatment of fruits with A, B and C

Table 1: Different biochemical compounds and their contents in pomegranate fruits under different sunscreen materials.

	RGR $gg^{-1}day^{-1}$	Fruit surface temperature (°C)	Anthocyanin's content		Antioxidant activity		Total phenol content	
			Juice (mg/ml)	Fruit skin (mg/g)	Juice (mg/ml)	Fruit skin (mg/g)	Juice (mg/ml)	Fruit skin (mg/g)
control	0.0166± 0.0[a*]	38 ± 1.38 [a]	0.1± 0.1 [a]	0.24± 0.027[c]	0.83± 0.2 [a]	0.81± 0.035[c]	9.9 ± 0.17[c]	18.2± 1.41[b]
activated kaolin (60 gL^{-1})	0.0168± 0.0 [a]	34.94± 0.45 [a]	0.11± 0.008 [a]	0.47 ± 0.026 [a]	1.67± 0.73[ab]	1.14± 0.012[a]	12.8± 2.1 [bc]	66.1± 3.17[a]
Quicklime (40 mlL^{-1})	0.0166± 0.0 [a]	35.61± 1.11 [a]	0.12 ± 0.005 [a]	0.34± 0.001[b]	2.86± 0.36[a]	1.07± 0.003[ab]	14.9± 0.61[b]	36.6± 5.55[b]
liquid paraffin (40 ml/fruit)	0.0165± 0.0 [a]	35.11± 0.27 [a]	0.13 ± 0.017 [a]	0.31 ± 0.002[b]	2.93± 0.07[a]	1.01± 0.009[b]	51.37± 1.54[a]	19.8± 10.74[b]

* The data represent mean ± Standard Error (SE) and the vertical bars with different letters are statistically significant.

Generally, according to the results obtained, the use of sunscreen compounds displayed the effect of lowering the temperature and increasing antioxidants compound thus the polyphenols and anthocyanins concentration increases. The amount of anthocyanin in the skin of the fruit is very significant in the treatment of kaolin. This is in accordance to the results of a study by Glenn *et al.,* (1999) that kaolin reduces sunburn and improves the size and color (anthocyanin) of the fruit.

Conclusion

In conclusion, activated kaolin is more effective than other sunscreens in preventing sunburn damage of pomegranate fruit Saveh Malas variety. Therefore, the effect of this substance on other types of pomegranate cultivars could be investigate in order to obtain high quality of fruits.

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