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RESEARCH PAPER

Identification and Characterization of Phenolic and Flavonoids Compounds Extracted from Tunisian Pomegranate Fruit Peel Exposed to Air Pollution: Gabes City, Tunisia

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ABSTRACT
The pomegranate (*Punica granatum*) fruit peel exposed to mixture air pollutants were collected from two sites with different air quality around the industrial area of Gabes city, Tunisia. The first site presented the 'Polluted site', which is situated in the oasis close to the industrial area. While, the second site referred to the 'Control site' located at 37 km from the industrial area. Using HPLC ES-MS, 21 phenols were identified and quantified in methanol extract from pomegranate fruit peel. The results showed that various phytochemical substances, including phenols acids and flavonoids, were identified and quantified in the peel extract. The polyphenols content and the flavonoids contents in peel obtained from polluted site were higher than that collected from the control site. The concentrations of the identified polyphenols were ranged between 0.39 and 7803.68 mg/ kg DW. The stimulation of some free phenolic compounds such syringic acid, transfrulic acid, epicatechin, rutin and quercetin was enregistred only in peel collected from contaminated environment. The quali-qualitative changes between sites are probably related to the difference in the air quality. The increase of polyphenols could be implicated during adaptive mechanisms under air pollution. Phenolic composition changes in *Punica granatum* peel could be also suggested as useful approach air pollution monitoring.

KEYWORDS: atmospheric pollution, defense, HPLC, oasis, polyphenols.

INTRODUCTION

The increase of industrial activities results in a significant concentration of air pollutants in plants. At high concentration, the air contaminants are harmful in plant metabolism causing oxidative stress (Chakrabati & Patra, 2015; Seyyednejed et al., 2017). To survive the contaminated environment, plants adopted numerous mechanisms. Several researchers reported that polyphenols can be used as antioxidant and played an important role in defense strategy to reduce air pollutants effects (Sytar et al., 2013; Ali et al., 2017; Pawlowska et al.,

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2019). Polyphenols includes two principal groups flavonoids and non-flavonoids (Zhou et al., 2019) which recently used as air pollution biomarkers (Khairallah et al., 2018; Mukherjee et al., 2019 ; Azzazy, 2020).

Pomegranate (*Punica granatum* L.) trees is one of the old deciduous fruit trees in the world, cultivated in several regions, which could tolerate abiotic stress (Teixeira da Silva et al., 2013). Pomegranates trees are largely produced in Mediterranean countries such as Tunisia, which is considered as an essential pomegranate secondary gene-center with more than 60 local ecotypes (Mars, 2001; Verma et al., 2010). About 40% of the total pomegranate production, is produced in Gabes oasis with the dominance of 'Gabsi' cultivar (Emna, 2010). Gabes oasis, where the pomegranate cv. Gabsi are cultivated, is exposed to several industrial air pollutants including gaseous and Particulate Matter (PM) with various heavy metals such as Zn , Cu and Pb (Tayibi et al., 2009; Taieb & Ben Brahim, 2014; Ben Atia-Zrouga et al., 2021). In fact, Gabes city includes a large industrial area including various industries. The industrial complex called the Tunisian Chemical Group (TCG) specialized in phosphate treatment, is considered as the biggest industrial park in Gabes and constitutes the major source of atmospheric industrial pollution in the region. Because of the intense industrial activities and the presence of several gaseous pollutants and particles, the region of Gabes was classified as the most polluted area in the Mediterranean basin (El Zrelli et al., 2015; Jeddi & Chaieb, 2019). Higher fluoride contents were enregistred in leaves of trees growing around the industrial area of Gabes. For example, fluoride concentration in pomegranate leaves was ranged from 60- 150 μg/g DW (Elloumi et al., 2016) .

In spite air pollution, Gabes oasis are the predominant producers of one of the most important cultivar. The cultivar 'Gabsi' is known for its high value and considerable sensory quality. It constitute an important source of polyphenols (Elfalleh et al., 2011). In general, it has medium weight and size (330 g, 70–80 mm), red-colored juice and mean juice yield more than 70 mL/ 100 g of arils (Zaouay et al., 2012). Pomegranate fruit is composed of seeds, juice, and peel. The peel represents about 50 % of total fruit weight (Gullon et al., 2016).

Thanks to its richness in bioactive compounds (Li et al., 2006; Hasnaoui et al., 2014), peels have been used in various applications (Silva et al., 2013; Gullon et al., 2016). For these reasons, some studies were conducted to analyze pollutants, such as, heavy metals in fruits peel (Dhimen et al., 2011; Stojanovic et al., 2017). Mallampati et al. (2015), found that avocado, hamimelon and dragon fruit peels, has the potential bio-adsorbents to extract dissolved pollutants in water.

Some studies were also done to identify the phytochemicals from pomegranate peel (Elfalleh et al., 2009; Abid et al., 2017; Kharchoufi et al., 2018), however, the information about the phenolic compounds profile of pomegranate fruit peel and their composition under industrial air pollution is scarce. To the best of our knowledge, this research is the first study carrying out to screening the phenolic compounds profiles pomegranate peel collected from their natural ecosystem under different air quality gradient.

Therefore, the objectives of the present study were (i) to determine the phenolic content including Total Phenolic Content (TPC) and Total Flavonoids Content (TFC) in pomegranate fruit peel collected from two different oasis around the industrial area of Gabes, and (ii) to identify and quantify the phenolic compounds profile through LC-ESI-MS.

MATERIALS & METHODS

Gabes is a city in southeastern east of Tunisia, covering an area of approximately 7100 km ^{2}, and is located between 33° 53' 17.077'' N latitudes and 10° 5' 51.079 E longitudes. The climate is arid with low average rainfall, an average annual temperature of 18.8 to 19.3°C. Easterly winds are predominant in Gabes region (Hamdi et al., 2014; Haj-Amor et al., 2020).

Punica granatum .L is an important horticultural crop and is at the heart of oasis environment. It was classified as sensitive species to air pollution, which reduced their total chlorophyll content and relative water content (Ben Abdalah et al., 2006; Zouari et al., 2017; Ben Amor et al., 2021). *Punica granatum* .L leaves exposed to air contaminant, showed morphological damages such as necrosis and chlorosis (Fig.1) (Ben Amor et al., 2021).

Figure 1. Necrosis and chlorosis observed in *Punica granatum* leaves collected around the industrial are of Gabes, Tunisia (Ben Amor et al., 2021).

The pomegranates were collected from the two studied oasis during September-November for two consecutive years. The two selected oasis were chosen at various distances from the factory. Oasis 1 called Chott-Essalem oasis (33°90'N, 10°09'E), was located close to the industrial park, it was considered as 'Polluted' Site (PS). The second one, called Zarat oasis (33°66'N, 10°35'E), was chosen at approximately 37 km from the factory, and considered as 'clean' or 'control site (CS). For each field site, five trees were sampled and 10 pomegranates were collected from 5 different trees.

For extract preparation, the pomegranate fruits peel was prepared by maceration using methanol (MeOH). Five replicates of each sample (1g of unwashed powdered peel) were extracted in 10 ml MeOH at room temperature on an orbital shaker (Stuart, Staffordshire, UK) for 2 hours at 200 rpm. The homogenate was centrifuged at 4°C**,** at 10,000 rpm for 15 min. The resulting supernatant was decanted, and the extract was kept in darkness and stored at −18°C until further application.

The Total Phenolic Content (TPC) was evaluated using the Folin-Ciocalteu method by a spectrophotometer (Tecan Infinite M200 Mannedorf, Switzerland) at the absorbance of 756 nm, as described by Singleton & Rossi (1965) with some modifications proposed by Gasmi et al. (2019). In each sample, the TPC were expressed in mg of gallic acid equivalent (GAE) per 1g of dry matter (mg GAE/g DW) through a calibration curve based on gallic acid.

The Total Flavonoids Content (TFC) was determined by the colorimetric method of aluminum chloride (AlCl3) (Veljkovic et al., 2013). From each peel extract, 1 ml was added to1 mL of a methanolic solution of AlCl3 (2%). After 10 min incubation at room temperature and using a spectrophotometer, the absorbance of the mixture was determined at 430 nm. Flavonoids concentrations were deduced from the curve calibration established with quercetin. The results were expressed as mg quercetin equivalents per 1 g of dry matter (mg QE/g DW).

The identification and the quantification of the polyphenolic patterns of pomegranate peel were determined using a liquid chromatography-electrospay ionization-tandem mass spectrometry (LC-ESI-MS) as described by (Rahmani et al., 2020). The pomegranate peel extract was filtered through a 0.45 µm membrane filter and then injected into the High Performance Liquid Chromatography system (HPLC). Quantification was obtained by injecting chemical standard solutions with known concentrations at the purity > 98%. The mobile phase was composed of a combination of two solvents: The first contains 0.1% formic acid in H2O and the second contains 0.1% formic acid in MeOH. The column temperature was set at 40 °C and the flow rate of the mobile phase was 0.4 mL/min. The results were expressed as mg/kg DW.

A one-way analysis of variance was performed to determine differences between studied sites, followed by Duncan test ($p \leq 0.05$) using IBM SPSS Statistics 25.

RESULTS AND DISCUSSION

As far as we know, there are no published data studying the variation of phenolic compounds under air pollution. For the first time, the pomegranate fruit peel cv. Gabsi was tested for its phenolic composition according to air pollution gradient.

Under stressful conditions such as industrial air pollution and for resisting, pomegranate trees develop various strategies such the increase of proline and soluble sugar contents (Ben Amor et al., 2018a). The accumulation of total phenolic compounds was also demonstrated as a defense strategy under different abiotic stress- (Sharma et al., 2019). In this study, The TPC (Fig.2) was significantly ($p \leq 0.05$) higher in pomegranate peel from polluted site than from control site (104.05 and 90.12 mg GAE/g DW for ‘polluted’ and ‘control’ sites, respectively). This findings confirm that pomegranate peel had a higher phenolic content (Elfalleh et al., 2009, 2012; Mansour et al., 2013).

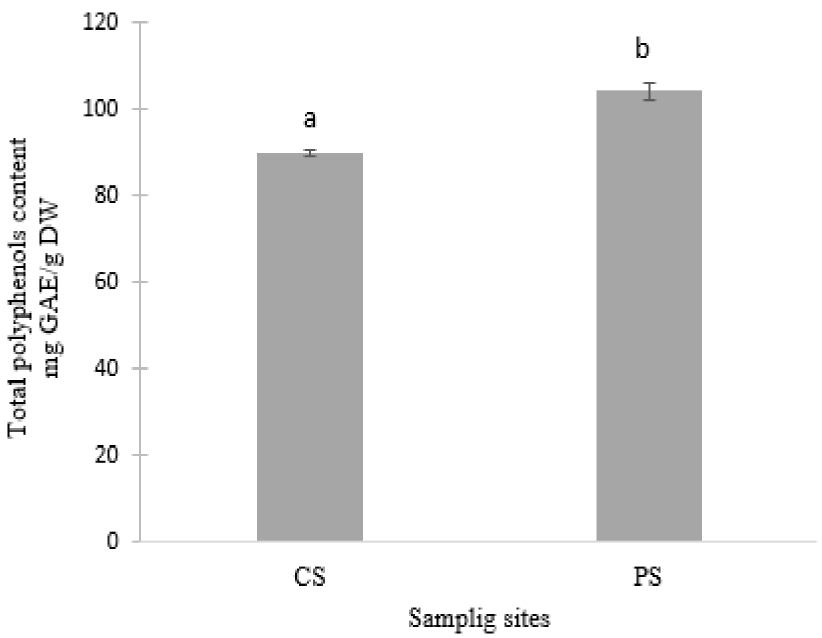

Figure 2. Total Polyphenols Contents (TPC) in pomegranate fruit peel collected from polluted and control site. The errors bars indicate mean ± SE (n=5). Different letters (a,b) indicate significant differences ($p < 0.05$; Duncan test) between sites.PS: polluted site; CS: Control site

The same pattern was observed in TFC (Fig.3). The concentration of total flavonoids measured in pomegranate fruit peel from 'polluted' site was 65.47 mg QE/g DW, while in control site, it was 51.51 mg QE/g DW. Significant differences ($p \leq 0.05$) were found between the sampling sites. This difference may be due to particles concentration deposition in pomegranate fruit peel. Researchers conducted in the same study sites showed that fluoride content in leaves of *Punica granatum* and *Phoenix dactylifera* collected at 37 km from the industrial area of Gabes were lower than those collected close to the factory (Elloumi et al., 2016; Ben Amor et al., 2018a,b).

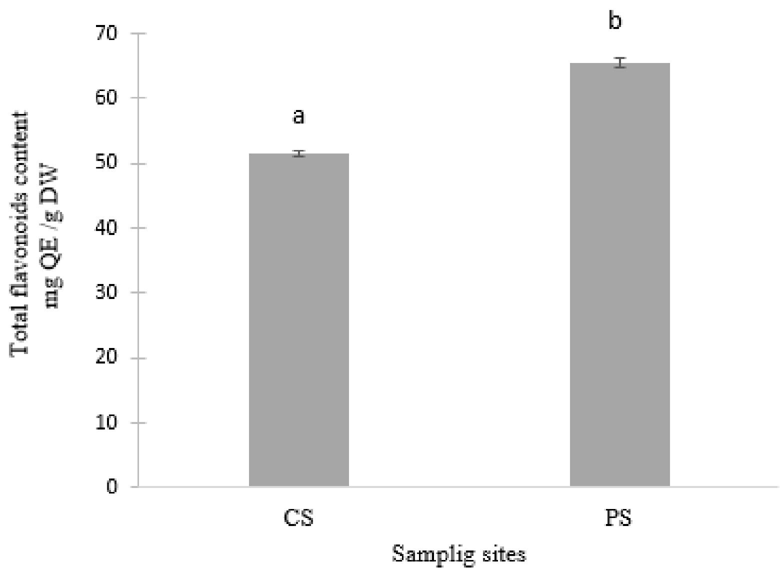

Figure 3. Total Flavonoids Contents (TFC) in Pomegranate fruit peel collected from polluted and control site. The errors bars indicate mean ± SE (n=5). Different letters (a,b) indicate significant differences ($p < 0.05$; Duncan test) between sites. PS: polluted site; CS: Control site

In this context, a positive correlation between the degree of environmental stress and the level of phenolic compounds accumulated in plants was found, suggesting their role in the defense mechanisms against stress (Boscaiu et al., 2010). The phenolic compounds accumulation was founded in different plant organs exposed to air pollution. In fact, it was observed that leaves of *Triticum aestivum* and *Betula pendula* exposed to air pollutants, increase their phenolic compounds content (Yamaji et al., 2003; Rai et al., 2007).

Moreover, the induction of compounds biosynthesis was demonstrated in responses to heavy metals pollution. This induction was found in maize in response to aluminum (Winkel, 2002), in wheat in response to nickel toxicity and in bean exposed to Cd2+ (Diaz et al., 2001).

Diaz et al., (2001) reported that *Phyllantus tenellus* leaves sprayed with copper sulphate accumulate more phenolic than the control leaves. This increase could be considered as a favorable adaptation as it might help in reducing the air pollutants effects. In fact, polyphenols has an important role in defense activity, which can scavenge ROS and chelate heavy metals (Ghnaya et al. 2013; Sytar et al. 2013; Ali et al. 2017).

Various research established that fruit peel have an important potential to reduce heavy metals such as Nickel (Ni) and lead (Pb) (Stojanovic et al., 2017).

Borycka and Zuchouski (1990) found that the decrease of cadmium and lead in aqueous solution is due to the presence of pomegranate peel. Phenolic compounds found to bind significant quantities of lead ions (Singleton et al., 1965).

The variation of TPC and TFC was related with the quali-quantitaive changes of individual antioxidants. The individual antioxidants found in the two extracts are shown in Tab.1. As expected, the composition of phenolic compounds in pomegranate peel from polluted and control sites were different. 21 antioxidants were identified with a high qualitative and quantitative variation from each extract. This variation is probably due to the air quality difference between the sampling sites. The concentrations of the identified polyphenols were ranged between 0.39 and 7803.68 mg/ kg DW. These compounds include two different classes:

Phenolic acids: quinin acid, gallic acid, protocatchuic acid, catechin acid, caffeic acid, syringic acid, transfluric, p-coumaric acid and 3.4-di-O-caffeoyquinic acid.

Flavonoids: epicatechin, rutin, Quercetrin (quercetin-3-o-rhamonoside), Naringin, Apegenin-7-o-glucoside , quercetin, kampherol, Naringenin, Apegenin , Luteolin, Cirsiliol and Acacetin

Quinic acid and gallic acid were the most abundant (4122.42 mg/ kg DW and 7803.68 mg/ kg DW) representing 71.43 and 86.40 % of total phenols. The characterization of gallic and catechin acids in methanolic extract of pomegranate peel was already identified by (Murthy et al., 2004; Elfalleh et al., 2009). P-coumaric acid was found to have the highest potential reduction of free radicals (Szwajgier et al., 2005). In this study p-coumaric acid content in pomegranate peel collected from PS (16.63 mg /Kg DW) is higher than in CS (6.82 mg /Kg DW).

Protocatchuic and 3.4-di-O-caffeoyquinic acids are absents in peel extract from PS, however syringic acid (m/z197) and transfluric acid (m/z193) were detected only in peel extract token from PS , and their amount was 16.62 % of total phenols.

Table 1. Phenolic compounds (mg/kg DW) identified in the pomegranate fruit peel collected from PS and CS by HPLC- LC-ESI-MS

				Polluted site (PS)		Control site (CS)	
	Code	**Phenolic Compounds**	**m/z**	**Rt (min)**	**CC (mg/ kg DW)**	**Rt (min)**	**CC (mg/ kg DW)**
Acids phenols	A1	quinic acid	179	2.10	7803.68	2.12	4122.42
	A2	Gallic acid	169	3.96	1029.36	3.97	489.65
	A3	Protocatchuic acid	153	-	N.D	6.88	25.37
	A4	Catechin (+)	289	11.11	140.58	11.10	11.33
	A5	Caffeic acid	179	14.49	7.88	14.81	4.27
	A6	Syringic acid	197	16.05	943.35	-	N.D
	A7	Trans frulic acid	193	23.09	15.96	-	N.D
	A8	3.4-di-O-caffeoyquinic acid	515	-	N.D	24.95	2.58
	A9	p-coumaric acid	163	21.05	16.63	20.91	6.82
Flavonoids	F1	Epicatechin	289	16.52	18.96	-	N.D
	F2	Rutin	609	23.97	16.88	-	N.D
	F3	Quercetrin (quercetin-3-o-rhamonoside)	447	-	N.D	27.09	116.67
	F4	Naringin	579	-	N.D	26.11	1.51
	F5	Apegenin-7-o-glucoside	431	27.24	9.95	27.24	4.23
	F6	Quercetin	301	32.05	1.16	-	N.D
	F7	Kampherol	285	32.08	2.96	32.11	2.43
	F8	Naringenin	271	34.12	0.65	34.24	0.44
	F9	Apegenin	269	34.68	0.41	34.67	0.39
	F10	Luteolin	285	35.09	1.86	35.04	1.48
	F11	Cirsiliol	329	35.84	1.40	35.84	1.25
	F12	Acacetin	283	-	N.D	40.44	0.40

Rt: retention time; CC: concentration: mg /kg DW ; N.D: not detected.

The stimulation of some free phenolic compounds such syringic acid, transfrulic acid, Epicatechin, Rutin and quercetin was enregistred only in peel collected from polluted site. It appears that phenolic compounds variation of pomegranate fruits peel could lead to develop new application for biomonitoring industrial atmospheric pollution. Also, the increase of phenolic compounds in pomegranate peels in responses of gaseous and dust pollution could reduce their negative impact. Therefore, the peel could be used for phytoremediation process.

CONCLUSION

The present study confirms the interest of pomegranate fruits peel as a good source of natural phenolic antioxidant, such as phenols acids and flavonoids, especially from polluted site. A significant difference in phytochemical identified and quantified in pomegranate fruit peel between polluted and control sites was found. This variation can serve as biomarker to air pollution.

The results showed also that the increase of TPC and TFC could play a key role to reduce air pollutants accumulation effects and serve as defense strategy to tolerate this stress.

GRANT SUPPORT DETAILS

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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