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Research Article

The Study of Phenolic Compounds Antioxidant Activity in Methanolic and Aqueous Extracts of Several Plant Species of Urmia Lake Margin

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ABSTRACT

This study was designed to examine *in vitro* antioxidant activity, scavenging capacity for total phenolic content, total flavonoid content, DPPH radical scavenging activity and superoxide (O_2^-) radicals inhibition assay for four plant species Urmia Lake Margin (*Alhaji camelorum, Chenopodium foliosum, Suaeda arcuata* and *Phragmites karka*). Plants were collected, their shoot were separated, and methanolic and aqueous extracts were prepared from shoot. The total phenolic and flavonoid contents were determined using the Folin-Ciocalteu method and a colorimetric method, respectively. The extracts were also evaluated on their reducing power assay and their capacity to scavenge for DPPH and O_2^- radicals. The results showed that the highest values for the total phenolic and flavonoid contents, DPPH, and O_2^- were related to methanolic extracts of samples *Alhaji*, *Chenopodium*, *Suaeda* and *Phragmites* respectively, which showed statistically significant differences ($p<0.01$). While the maximum values for the reducing power assay was related to aqueous extracts of sample *Chenopodium* ($p<0.01$). Findings suggest that the *Chenopodium* sample is more successful than other samples. Moreover, it was found that methanol was more successful in extraction procedure than aqueous ($p<0.01$).

Key Words: Antioxidant activity, Phenolic compounds, *Alhaji, Phragmites, Chenopodium, Suaeda*

INTRODUCTION

Phenolic compounds are potentially synthesized by all plant cell type[1]. Phenolics are antioxidants with redox properties, which allow them to act as reducing, hydrogen donators, and singlet oxygen quenchers[2]. Antioxidants are important because they have the ability of protecting organisms from damage caused by free radical-induced oxidative stresses[3]. Free radical reactions are involved in many biological processes that cause damage to lipids, proteins, membranes and nucleic acids, thus giving rise to a variety of diseases[4-6]. Among the plant compounds that there has antioxidant properties, phenolic compounds distributed widely in many plants. Antioxidant properties phenolic compound is mainly due of the reduction and their chemical structures that enable them to neutralize free radicals, Phenolic compounds through electron donation to free radicals, inhibit lipid oxidation reactions[7]. The need for antioxidants becomes even more critical with increased exposure to free radicals. Pollution, cigarette smoke, drugs, illness, stress and even exercise can increase free radical exposure. Because so many factors can contribute to oxidative stress, healthy lifestyle and a well-balanced, wholesome diet, antioxidant supplementation is now being recognized as an important means of improving free radical protection[8]. Most of the ant oxidative potential of plant foods, which could be beneficial to human health, is due to the properties of phenolic compounds. Reactive oxygen species (ROS) are produced in all aerobic cells as by-products of oxygen metabolism. When ROS generation overwhelms the cellular antioxidant capacity, oxidative stress ensues. Under these conditions, ROS can oxidize lipids, proteins and nucleic acids, ultimately leading to cell death or transformation. Phenolic compounds can act as reducing agents, free radical scavengers, hydrogen donators and inhibitors of pro-oxidative enzymes[9,10]. The previous studies showed that *Alhagi maurorum* contained many secondary metabolites including flavonoids, fatty acids, coumarins, glycosides, sterols, steroids, resins, vitamins, alkaloids, carbohydrates, tannins, unsaturated sterols and triterpenes. It exerted antibacterial, anti-inflammatory, antipyretic, analgesic, antioxidant, gastrointestinal, cardiovascular, diuretic, and dermatological and many other effects. The present review will highlight the chemical constituents and the pharmacological and therapeutic effects of *Alhagi maurorum*[11]. *Phragmites karka*, a perennial halophytic grass, usually grow as pure population in flooded saline habitats[12] could attain a height of about 5-7 meters with rapid growth rate[13]. This plant species is traditionally used as a remedy for diabetes, and also known for its diuretic properties[14]. *Phragmites karka* is capable of producing lignocellulosic biomass for bio-ethanol production[15-17]. The consideration that plants with high lignocellulosic content are promising sources of antioxidant compounds[18] makes *Phragmites karka* an interesting candidate for research on this aspect. Further, it is also reported that production of secondary metabolites could be increased with increasing salt concentration of growth medium[19,20]. The inhibitory

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effect of phenolic compounds in the *Phragmites* in a series of advanced cancer cell characteristics observed[21]. Mainly these antioxidants are powerful allies in combating inflammation and lowering heart disease and cancer risk. Flavonoids and other plants phenolics, phenolic acids, stilbenes, tannins, lignin, are especially common in leaves, flowering tissues, and woody parts such as stems and barks[22]. *Chenopodium foliosum* Asch. is an annual herb growing in Europe, North Africa, Central and South-West Asia, as well as occasionally naturalized in other regions[23]. This plant has also been known in Bulgarian folk medicine as "garliche" or "svinski yagodi" (swine's berries). The decoction of its aerial parts has been used for treatment of cancer and as an immunostimulant and antioxidant and the plant has been recognized by Bulgarian legislation as a medicinal plant[24,25]. It seems that the polyphenols in different species *Chenopodium*, large effects on health, they including antioxidants activity, apoptotic anti-inflammatory activity, improved endothelial function and inhibition of angiogenesis and cell proliferation[26]. *Suaeda monaica* Forssak.ex.Gmel belonging to Chenopodiaceae family is a salt marsh mangrove herb similar to *Suaeda maritima* in appearance. It is a herb, smaller in size. Leaves simple, succulent, linear, young twigs are slender ribbed. The leaves have been used as edible green leaves. Traditionally, the leaf from *Suaeda monaica* is known to use as a medicine for hepatitis and scientifically it is reported to be used as ointment for wounds and possess antiviral activity, because of the presence of triterpenoids and sterols[27,28]. In present the research total phenolic content, total flavonoid content, total antioxidant capacity and 1, 1-diphenyl-2 picryl hydrazyl (DPPH) and superoxide (O_2^-) radicals scavenging activity was measured for four plant species of Urmia Lake Margin.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Samples and extracts preparation

Four plant species (*Alhaji camelorum*, *Chenopodium foliosum*, *Suaeda arcuata* and *Phragmites karka*) were collected in July-August-2014 from Urmia Lake Margin in northwestern Iran. Their shoots were separated and then they were dried at room temperature and reduced to fine powder. The air- dried ground (30 g) was extracted with each of the solvents (pure methanol and water) (100 ml) in a soxhlet apparatus at 60˚C for 30 min. The samples were centrifuged for 20 min at 4000g. The supernatant was filtered through filter paper and stored at 4˚C until analysis for one week[29].

Total phenolic content assay

Total phenolic content of the extracts was determined using the Folin-Ciocalteau reagent Horwitz (1984). Folin-Ciocalteau reagent was diluted 10 times with distilled water. The extract solution (50 µL) was mixed with 1ml diluted Folin-Ciocalteau reagent, 0.5 ml sodium bicarbonate solution (2%), and 0.95 ml distilled water. The mixture was incubated at room temperature for 30 min. The absorbance of the solution was determined at 750 nm using a spectrophotometer (Bio wave, S 2100, U K). The total phenolic content was expressed as mg gallic acid equivalents (GAEs) per gram of extract[30].

Determination total flavonoid content

Flavonoid content was determined according to the method described by Zhishen *et al*. (1999). An appropriate dilution (1 ml) of the extract was mixed with 0.3 ml of $NaNO_3$ (5%) and 4 ml water. 0.3 ml $AlCl_3$ (10%). The mixture was allowed to react for 6 min and was mixed with 2 ml of NaOH (1 M) and finally balloon containing 10 ml of solution volume reached and the absorbance was read at 430nm against a blank sample without reactants. The total flavonoid content was determined by using a quercetine standard curve and stated as the mean [mg quercetine equivalents (QEs) per 100g of extract] ± SE for the triplicate extracts[31].

Reducing Power Assay (RPA)

The reducing Power Assay (RPA) of the extracts was assessed as described by Yildirim *et al*. (2001). Fifty µl of extracts were added to 2.5 ml phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1%). Then 2.5 mL Trichloroacetic acid (10%) was added to the mixture and the mixture incubated at 50°C for 20 min. The supernatant (2ml) was mixed with 2 ml distilled water and 0.5 mL ferric chloride (0.1%), which was then centrifuged at 650 *g* for 10 min. The absorbance was read at 700 nm[32].

Superoxide radical inhibition percentage (O_2^-)

The method described by Jing *et al*. (1995) was used to determine O_2^- radical scavenging activity of samples. One ml of extract was added to 9 ml of HCl-Tris buffer (pH 8.2, 50 mM per L), then test tubes incubated in water bath at 25°C for 20 minutes. 40 µl of pyrogallol solution (45 mM per L pyrogallol in 10 mM per L HCl) was added to the mixture. The mixture were mixed at 25°C for 3 min. Then a drop of ascorbic acid (0.008%) was added in it[33]. The absorbance of the reaction mixture was determined after 5 minutes at 420 nm that it was refers on pyrogallol oxidation rate. O_2^- radical scavenging % = $[A_0-(A_1-A_2)/A_0] \times 100$

DPPH radical scavenging activity

The measurement of DPPH radical scavenging activity was carried out according to the method of Barros *et al*. (2007). A total of 20 µl of extract was added to 2 ml of methalonic DPPH (0.0023) solution. The mixture was incubated in room temperature for 1 hour before the change in absorbance at 517 nm was measured[34]. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation: DPPH radical scavenging % = $[(A_0 - A_1)/A_0] \times 100$ Where A_0 is the absorbance of the DPPH solution and A_1 is the absorbance of the sample.

Statistical analysis

All experiments were performed in triplicate (n=3) and results were expressed as mean±SEM. Statistical analyses were carried out with (SPSS package version 16.0) using one-way analysis of variance (ANOVA). Significant differences were calculated according to the Tukey's test.

The significant difference was statistically considered at the level of $p<0.05$.

RESULTS

Total phenol content

In this study, phenolic compounds from shoot for four plant species (*Alhaji camelorum, Chenopodium foliosum, Suaeda arcuata* and *Phragmites karka*) were measured using 2 solvents, water and methanol. The total contents of phenolic compounds in the 4 plant species are reported in table 1. As the phenolic substances make one of the major groups of compounds acting as primary antioxidants or free radical scavengers, it was reasonable to determine their total amount in the selected plant extracts. The total content of phenolic compounds in the extracts was determined by the Folin-Ciocalteau method. High values total phenolic content of both the methanolic and aqueous extracts were observed respectively, in *Alhaji* (33.63 ± 0.68 and 21.92 ± 0.28 mg GAEs/g extract) and lower values in *Chenopodium* aqueous extract and *Phragmites* methanolic extract, respectively (27.01 ± 0.28 and 10.9 ± 0.73 mg GAEs/g extract). Table 1 shows that the methanolic extract of *Alhaji* had the highest total phenolic content, so it had the highest antioxidant activity. The average of total phenol content in 4 plants species of methanolic and aqueous extract was 29.89 ± 0.97 and 13.96 ± 1.41 mg GAEs/g extract, respectively. Table 2 showed statistically significant differences. Variance analysis of data showed that 4 species for total content of phenolic compounds contents is significant ($p<0.01$, Table 2).

Determination of total flavonoid content

Table 1 shows the flavonoid values obtained from the 4 plants species. High levels total flavonoid content of methanolic and aqueous extracts were found respectively, in *Chenopodium* (15.77 ± 0.96 mg QEs/100 g extract) and *Alhaji* (5.94 ± 0.68 mg QEs/100 g extract) and lower levels in *Alhaji* (4.42 ± 0.1 mg QEs/100 g extract) and *Suaeda* (3.67 ± 0.11 mg QEs/100 g extract). This indicates that the total flavonoid content of methanolic extract of *Chenopodium* was higher than aqueous extract of species. The average of total flavonoid content in 4 plants species of methanolic and aqueous extract was 10.4 ± 1.56 and 4.91 ± 0.3 mg QEs/100 g extract, respectively. Table 2 showed statistically significant differences. Variance analysis of data showed that 4 species for determination of total flavonoid content is significant ($p<0.01$, table 2).

Reducing power assay

The amount of reducing power was measured spectrophotometrically at 700 nm. Table 1 showed the reducing ability of different solvent extracts. Absorbance of the solution was increased when the concentration increased. A higher absorbance indicates a higher reducing power. Highest reducing power assay of methanolic and aqueous extracts obtained respectively, in *Suaeda* (0.613 ± 0.05), and *Chenopodium* (0.646 ± 0.008) and lowest obtained in *Phragmites* (0.420 ± 0.04) and *Suaeda* (0.490 ± 0.01). However, reducing power of aqueous extracts of *Chenopodium* (0.646 ± 0.008) was higher than the methanolic extracts. The average of reducing power assay in 4 plants species of methanolic and aqueous extract was 0.543 ± 0.02 and 0.572 ± 0.02 sample, respectively. The reducing power of 4 plants species increased significantly according to their phenol content. Table 2 showed statistically significant differences. In case of reducing ability (700 nm), a significant difference was observed between the plants and solvent ($p<0.01$, Table 2).

Superoxide radical inhibition percentage (O_2^-)

In table 1 of present study, percentage highest and lowest of O_2^- scavenging capacity of both aqueous and methanolic extracts were obtained in *Phragmites* (71.07 ± 7.43% and 83.04 ± 1.21%), and the lowest value were distinguished in *Alhaji* (39.68 ± 6.02%) and *Chenopodium* (2.12 ± 1.28%), respectively. These results clearly suggest that the antioxidant activity of methanolic sample is also related to its ability to scavenge O_2^-. Maximum O_2^-scavenging percentage was for *Phragmites* (71.07 ± 7.43%) and the minimum O_2^- scavenging percentage was for *Chenopodium* (2.12 ± 1.28%)**.** The average of O_2^- inhibition percentage in 4 plants species of methanolic and aqueous extract was 30.33 ± 9.59% and 56.15 ± 4.27% sample, respectively. Table 2 showed statistically significant differences. Variance analysis of data showed that 4 species for superoxide radical inhibition percentage is significant ($p<0.01$, Table).

DPPH radical scavenging activity

The DPPH radical scavenging activity (%) in each investigated sample are shown in table 1. The highest and lowest DPPH radical scavenging ability of methanolic extracts was found in *Suaeda* (32.17 ± 3.7%) and *Alhaji* (15.03 ± 3.11%), and highest and lowest DPPH radical scavenging ability of aqueous extracts observed in *Suaeda* (28.97 ± 1.05%), and *Chenopodium* (11 ± 2.25%), respectively. The results demonstrate that the most active radical scavenger was the methanolic extract from *Suaeda* (32.17 %) and the lowest scavenging activity was observed for the aqueous extract from *Chenopodium* (11 ± 2.25%). The average of DPPH radical scavenging activity in 4 plants species of methanolic and aqueous extract was 21.63 ± 3.19% and 17.85 ± 2.13% sample, respectively. Table 2 showes statistically significant differences. Variance analysis for DPPH radical scavenging activity, no significant difference was shown between the plants and solvent ($p>0.05$, Table 2).

DISCUSSION

Bang and *et al*. 2007 have shown that the use of methanol increase the extract phenolic content and even the concentration of methanol can be affect in the extraction of plant compounds[35]. In this study, the 2 methanol and aqueous solvent were used and influence both solvent evaluated the extraction compounds. As a result of this study was observed that methanol solvent was more successful than aqueous solvent in extraction procedure. The results could be used for further study.

Total phenolic content assay

Table 1. Comparison of phenol content (mg GAEs/g extract), flavonoid content (mg Qes/100 g extract), reducing power, DPPH radicals and superoxide scavenging for four plant species of Urmia Lake Margin.

Solvent O_2^- (%)	Sample	Phenol content[a]		Flavonoid content[a] (absorbance)	Reducing power[b]	DPPH (%)
		(mg GAEs/g extract)	(mg Qes/100 g extract)			
Aqueous Extract	*Alhaji camelorum*	21.92 ± 0.28	5.94 ± 0.68	0.620 ± 0.04	14.30 ± 1.40	39.68 ± 6.02
	Phragmites karka	10.94 ± 0.12	5.31 ± 0.22	0.533 ± 0.008	17.14 ± 0.63	71.07 ± 7.43
	Chenopodium foliosum	10.9 ± 0.73	4.72 ± 0.3	0.646 ± 0.008	11 ± 2.25	52.1 ± 2.59
	Suaeda arcuata	12.1 ± 0.8	3.67 ± 0.11	0.490 ± 0.01	28.97 ± 1.05	24.76 ± 3.57
Methanolic Extract	*Alhaji camelorum*	33.63 ± 0.68	4.42 ± 0.1	0.593 ± 0.03	15.03 ± 3.11	83.04 ± 1.21
	Phragmites karka	27.01 ± 0.28	6.19 ± 0.44	0.420 ± 0.04	16.58 ± 1.68	2.12 ± 1.28
	Chenopodium foliosum	27.89 ± 2.47	15.77 ± 0.96	0.546 ± 0.01	22.76 ± 1.4	2.12 ± 1.28
	Suaeda arcuate	31.04 ± 0.79	15.21 ± 0.27	0.613 ± 0.05	32.17 ± 3.7	11.39 ± 5.14

[a]Each value is expressed as mean standard error (Mean ± S.E, n = 3), $p < 0.05$.
[b]Each value is expressed as mean standard deviation (Mean ± S.E, n = 3), $p < 0.05$.

A strong correlation has been observed between the phenols and antioxidant activity. Also strong relationship between total phenolic content and antioxidant activity has been reported by[36-38] do not find any such kind of correlation between antioxidant activity and phenolic content in plant extracts. Phenolic compounds are secondary metabolites, widely distributed in plants. They are important components of many fruits and vegetables not only for their major influence on sensory qualities of the fruit (color, flavor, and taste), but also for their antioxidant, anticarcinogenic, antimicrobial, antiallergic, antimutagenic, and anti-inflammatory properties[39]. The total phenolic content in *Dalbergia latifolia* bark of alcohlic extract had higher level of phenolic compounds, that is agrees with our results[40]. Although, the total phenolic content in our experiment higher than this study. The one study, the lowest average amount of total phenolic compounds was found in *Phragmites australis*, that it in agreement with our results[41]. In black sorghum seeds observed higher content of total phenols, while our study *Phragmites* showed the lowest valus[42]. The highest total phenolic content was found of the methanol extract of *Suaeda monaica,* that in agreement with our study[43]. Also, in a study the total phenol content was found of *Chenopodium album*, that is in contrast our study[44]. The results from this study suggested that phenolics are important components of these plants.

Determination of total flavonoid content

Phenols are very important plant constituents with multiple biological functions including antioxidant activity because of their radical scavenging ability due to their OH groups. The presence of flavonoids might be responsible for the antioxidant activity of the plants. In very recent years, flavonoids being potent free radical scavengers have attracted a tremendous interest as possible therapeutics against free radical mediated diseases[45]. It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of the actions of flavonoids are through scavenging or chelating processes[46,47]. The compounds, such as flavonoids, which contain hydroxyl groups, are responsible for the radical scavenging effect in the plants[48,49]. According to our study, the contents of these phytochemicals in extract can explain its antioxidant activity. The total flavonoid content in *Dalbergia latifolia* bark of methanolic extract had higher level of phenolic compounds, that is agrees with our results[50]. Although, the total flavonoid content in our experiment higher than this study. The one study, the total flavonoid content was found the methanolic extract of *Suaeda monaica*, that is agrees with our results[51]. One other study, the total flavonoid content was found of *Chenopodium album*, that it in contrast our study[52].

Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity[53]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants[54]. Among the solvent tested, methanol extract of *Suaeda monaica* exhibited higher reducing activity, that it is in contrast our study[55]. The reducing capacity of *Dalbergia latifolia* bark of alcohlic extract had higher level of phenolic compounds, that in contrast with our results[56]. Also, in a study the reducing power assay was found of *Chenopodium album*, that it in contrast our study. The one study, the reducing power assay was found of *Centepede* grass and *Festuca* samples, that it in agreement our study[57].

Table 2: Variance analysis of phenol content (mg GAEs/g extract), flavonoid content (mg QEs/100 g extract), reducing power, DPPH radicals and superoxide scavenging for four plant species of Urmia Lake Margin.

Source	df	Phenol content (mg GAEs/g extract)	Flavonoid content (mg QEs/100 g extract)	Reducing power (absorbance)	DPPH (%)	O_2^- (%)
Plant	3	** 98.886	*39.311	**0.021	**319.032	**3138.958
Solvent	1	**1522.112	**180.621	^{ns}0.005	^{ns}85.730	** 4000.034
P * S	3	**14.002	**69.011	**0.018	^{ns}46.026	**1366.278
Error	16	3.221	0.693	0.003	53.680	65.178

P * S: Plant * Solvent; Df: degrees of freedom; **: Significant differences $p<0.01$; *: Significant differences $p<0.05$; Ns: no Significant differences $p>0.05$

Superoxide radical inhibition percentage (O_2^-)

ROS may be very damaging, since they can attack lipids in cell membranes, protein in tissues or enzymes, carbohydrates and DNA, to induce oxidations, which may causes cancer, atherosclerosis, aging, immune suppressant, inflammation, ischemic heart disease, diabetes, hair loss and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease[58,59]. Superoxide anion plays an important role in the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induces oxidative damage in lipids, protein, and DNA[60,61] Superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals[62]. In a study, the O_2^- scavenging activity of *Suaeda monaica* was indicates that is higher than our O_2^- scavenging effect[63]. In a study, the results of *in vitro* antioxidant data showed a significant O_2^- scavenging of hydro alcoholic extract of *Cynodon dactylon*, that it in agreement our study[64]. In other study, the results of in vitro antioxidant data showed high O_2^- scavenging of *Trifolium pratense* L, that it in agreement our study[65]. Table 2 showes statistically significant differences.

DPPH radical scavenging activity

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[66]. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The experimental data of the extract revealed that the extract is likely to have the effects of scavenging free radicals. From the result, in the present study a dose dependent relationship in the DPPH radical scavenging activity. It has been shown that the scavenging effects on the DPPH radical increases sharply with the increasing concentration of the samples and standards to a certain structure[67], and hence are said to be strongly dependent on the extract concentration. DPPH value was significantly higher in *Chenopodium quinoa* seeds that it is in contrast to our study[68]. Previously published data have indicated that the antioxidant activities of phenolic plant compounds are correlated with anticancer and anti-atherosclerotic potential[69]. The lowest scavenging activity was observed for the extract from *Phragmites australis*[70], that in contrast with our results. The DPPH radical scavenging activity in *Dalbergia latifolia* bark of methanolic extract had higher level of our DPPH radical scavenging activity[71]. In a study, among the solvent tested, methanol extract of *Suaeda monaica* exhibited highest DPPH radical scavenging activity that, it in agreement our study[72].

CONCLUSIONS

Alhaji camelorum, Chenopodium foliosum, Suaeda arcuata and *Phragmites karka* phenolic extract possess antioxidant activity, which might be this phenolic extract helpful in preventing or slowing the progress of various oxidative stress-related diseases. Methanolic extract from 4 plant species Urmia Lake Margin showed a strong antioxidant activity. However, to use the extracts of these phenolic compounds as antioxidant in foods, methanol should be substituted with some harmless solvent. Although water is not as effective as organic solvents to extract useful compounds from plants by-products. Further investigation on the isolation and identification of antioxidant component(s) in the different almonds genotypes may lead to chemical entities with potential for clinical use.

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