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In vitro assessment and comparative efficacy on antioxidative effects of petroleum ether and methanol extract of different citrus fruit peels in Bangladesh

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Abstract

The prevention of oxidative stress caused by microorganisms or other adverse environmental stimuli is not clarified well. The current study has been undertaken regarding this phenomenon along with petroleum ether and methanol extraction of five varieties of citrus fruit peels, *C. limittoids*, *C. hystrix*, *C. medica*, *C. reticulate* and *C. lemon*. For total antioxidant capacity during petroleum ether extraction, different concentrations of peel extract were used. *C. reticulate* and *C. medica* were found to be potential showing higher antioxidant capacity and the absorbance were increased dose dependently when compared to catechin. Other varieties also showed a increased absorbance indicating higher antioxidative effects. Similarly, the extracts of peels after methanol treatment were found to have a higher absorbance where the absorbance was increased dose dependently and *C. hystrix* and *C. limittoids* showed potent effect. Other varieties were shown to have increased antioxidative effects because of their increasing absorbance. The reducing power capacity during petroleum ether extraction was enhanced dose dependently while *C. reticulate* and *C. medica* showed higher absorbance showing higher reducing power capacity. The absorbance of different concentrations of extract in methanol was examined and the values were increased similarly with increasing concentrations. *C. reticulate* and *C. medica* showed higher effect when compared to ascorbic acid. Other varieties also showed enhanced effect and the effects were assumed to be potential in both petroleum ether and methanol extract. Therefore, all five varieties are recognized to have bioactive compounds with diverse clinical importance in response to adverse biotic or abiotic stress.

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Introduction

Antioxidants are the chemical substances that reduce or prevent oxidation and have the ability to counteract the damaging effects of free radicals in tissues and thus are believed to protect against cancer, heart disease and several other diseases (Zhang *et al*., 2015). Antioxidant compounds in food play an important role as a health-protecting factor. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes and phenolic acids have been recognized as having the potential to reduce disease risk. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species (ROS) are made in the biological systems from a wide variety of sources caused by adverse environmental situation (Krishnamurthy and Rathinasabapathi, 2013). These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases. It is well known that citrus fruits have been shown to play the potential role on the prevention of oxidative stress caused by the environmental stress either biotic or abiotic. Therefore, it is assumed that some of the compounds are present in the citrus fruits and play the critical role regarding this phenomenon although the mechanism is not clarified well.

Citrus fruits such as orange, lemon and lime, have been widely cultured and processed into juice. During the manufacture of citrus juice, very large amounts of byproduct wastes, such as peels are formed every year. These peels are believed to exhibit a broad spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Ortuno *et al*., 2006). Various types of citrus fruits such as lemons, oranges, limes and grapefruits are recommended to be responsible for the prevention of degenerative disease because of the availability of important nutrients. Vitamin C, folic acid, dietary fibers, carotenoids, potassium, selenium and a wide variety of phytonutrients are available in citrus fruit. Many chemical compounds such as alkaloids, flavonoids, glycosides, saponins, resins, oleoresins, sesquiterpene, phenolic compounds, fats and oils are reported to be present in citrus medicinal plants (Dhanavade *et al*., 2011).

Citrus byproducts, if utilized fully, could be major sources of phenolic compounds. The peels, in particular, are an abundant source of natural flavonoids and contain higher amount of phenolics compared to the edible portions. It has been reported that the contents of total phenolics in peels of lemons, oranges, and grapefruit were 15% higher than those in the peeled fruits (Sawalha *et al*., 2009). Flavonoids in citrus are a major class of secondary metabolites. The peel contains the higher amount of flavonoids than other parts of the fruits and is involved in playing the vital biological and biochemical functions (Sawalha *et al*., 2009). Because of the presence of large amount of phytonutrients in citrus peel, they are believed to play a vital role on the prevention of diverse complications caused by the environmental adverse stimuli either biotic or abiotic. These adverse stimuli or stresses cause severe effects in the biological system and produce cellular damage and impairment of biological and biochemical functions.

Recently known some potential compounds are identified in citrus fruits and peels which are recognized to be involved in prevention of oxidative cellular damage thereby the cells survive in the atmosphere. Therefore, it is substantial to make strategy to find the citrus species and to identify the compounds present in the species and peels. The mechanism of these compounds on prevention of oxidative damage is yet to be identified. These compounds are referred to as antioxidant molecules and show antioxidative effects thereby a major and potential aspect in the prevention of oxidative stress caused by adverse environmental stimuli. Moreover, recent investigations reveal that citus fruit peels are involved and shows a potential on antioxidative

effects (Al-Juhaimi, 2014; Divya *et al*., 2016). Based on several lines of evidences, it is assumed that lemon peel is a major phytonutrient essential for the prevention of different complications and shows clinical importance. Therefore, the current study involves the selection of some fruit species and the effectiveness of lemon peel of different varieties on anti-oxidative effects. The purpose of the current study is to explore the comparison between the antioxidative effects of petroleum ether extract and methanolic extract of five Bangladeshi citrus fruits peel.

Materials and methods

Plant materials

The fresh unriped five citrus fruits, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) were collected from citrus research centre, Jointiapur, Sylhet and the garden of local farmer to get chemically treated free sample. All the samples were peeled off, dried, grinded into coarse powder and were extracted with petroleum ether for fat free and methanol under sonication bath for highest yield of extracts.

Local names of citrus species: So = Sorbotilebu; Sat = Satkora; Ja = Jaralebu; Kh = Khasikomla and Le = Lebu

Extraction procedure

Dried peel powder of different citrus samples were taken in glass bottle containing plastic cap and extracted initially with petroleum ether under sonication bath (Trans sonicator, T-60) to remove the fatty constituents of peel. The sample was extracted by three times to get the maximum extract.

The mixture (sample + solvent) was filtered through Whatman No.1 filter papers. The filtrate was then concentrated with a rotary evaporator under reduced pressure at 50 °C to obtain brownish mass of peels. After getting the petroleum ether treated peel extract, residue peel powders were allowed to dry and were dissolved in methanol for further extraction with the same process as mentioned above.

In vitro assay of total antioxidant capacity

The total antioxidant capacity of different citrus peel extracts, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) was determined according to the procedure of Prieto *et al.* (1999) with some modifications. Briefly, 0.3 mL solutions of different extracts or standard (catechin) at different concentrations (100, 200, 300, 400 and 500 µg/mL) were taken in the test tubes. 3.0 mL of reaction mixtures containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate were added into each of the test tubes. The test tubes were incubated at 95 °C for 10 min to complete the reaction. The absorbance of the solutions was measured at 695 nm using spectrophotometer against blank after cooling at room temperature. A typical blank solution contained 3.0 mL of reaction mixtures and the appropriate volume (300 µL) of the same solvent used for the sample and it was incubated at 95 °C for 10 min and the absorbance was measured similarly at 695 nm.

In vitro assay of reducing power capacity

The reducing power capacity of different extracts, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) was evaluated by the method described by Oyaizu (1986). Briefly, 0.25 mL solutions of different extracts or standard (ascorbic acid) at different concentrations of solution (20, 40, 60, 80 and 100 µg/mL) were taken into the test tubes. 0.625 mL of potassium phosphate buffer (0.2 M) (pH 6.6) and 0.625 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%) solution were added into each of the test tubes. The reaction mixtures were incubated for 20 min at 50 °C to complete the reaction. 0.625 mL solution of 10% trichloro-acetic acid (TCA) was added into each of the test tubes. The total mixtures were centrifuged at 3000 rpm for 10 min. 1.8 mL supernatant was withdrawn from the mixture and mixed with 1.8 mL of distilled water. 0.36 mL solution of 0.1% ferric chloride ($FeCl_3$) was added to the diluted reaction mixtures. Then the absorbance of the solutions was measured at 700 nm using a spectrophotometer against blank. A typical

blank solution contained the same solution mixture without plant extract or standard and it was incubated under the similar conditions as done for the sample solution. The absorbance of the blank solution was measured at 700 nm against the solvent used in solution preparation. The increased absorbance of the reaction mixture indicated the increase reducing power capacity of the sample extract.

Results

Effects of different concentrations of peel extracts treated with petroleum ether on total antioxidant capacity

Total antioxidant capacity of peel extract of different varieties of fruits was determined spectrophotometric ally and the results were compared with standard katechin. Different concentrations of peel extracts (100, 200, 300, 400 and 500 µg/mL) of different varieties of fruits were used in the assay and the absorbance were recorded. Fig. 1 shows the total antioxidant capacity of petroleum ether extract of citrus peel, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le). For *C. limittoids* (So), the absorbance of the extracts of different doses (100, 200, 300, 400 and 500 µg/mL) were recorded as 0.031, 0.063, 0.093, 0.122 and 0.173 respectively while for *C. hystrix* (Sat) variety, the following absorbance were recorded as 0.042, 0.081, 0.112, 0.142 and 0.181 respectively for the above concentrations of the test extract. Similarly, the absorbances for C. medica (Ja) variety were found as 0.052, 0.121, 0.161, 0.191 and 0.233 respectively for different concentrations of extract. The effects of different concentrations (100, 200, 300, 400 and 500 µg/mL) of standard catechin on total antioxidant capacity were demonstrated in Fig. 1. The absorbance was increased dose dependently and the values were recorded as 0.13, 0.23, 0.26, 0.33 and 0.37 respectively.

Table 1. Comparative efficacy on total antioxidant capacity of petroleum ether and methanol extract of different varieties of citrus fruit peel. The effects of 500 µg/mL peel extracts of different varieties of fruit were shown. The effects of catechin (control) on total antioxidant capacity were similarly done except giving peel extract. The results are means of ± standard deviation for three values in each group of sample.

Name of sample	Total antioxidant capacity (absorbance at 695 nm)	
	Petroleum ether (PE) fraction	Methanol (Me) fraction
Catechin	0.370 ± 0.01	0.370 ± 0.01
C. limittoids (So)	0.173 ± 0.0005	0.204 ± 0.001
C. hystrix (Sat)	0.181 ± 0.0005	0.320 ± 0.01
C. medica (Ja)	0.233 ± 0.0003	0.138 ± 0.001
C. reticulate (Kh)	0.322 ± 0.001	0.177 ± 0.001
C. lemon (Le)	0.232 ± 0.001	0.120 ± 0.01

The results showed that the total antioxidant capacity had increased gradually in presence of the increased concentrations of the peel extracts. Among the three different varieties, the higher absorbance was recorded for *C. medica* (Ja) when compared to the control catechin however the values were lower than catechin.

Other varieties of extracts of traits also showed the potent antioxidant capacity because of their increasing absorbance when compared to standard catechin. The total antioxidant capacity were examined for another two varieties, *C. reticulate* (Kh) and *C. lemon* (Le) where the absorbance were found as 0.097, 0.172, 0.232, 0.282 and 0.322 respectively for *C. reticulate* (Kh) variety for the similar doses of extracts of peels. For *C. lemon* (Le) variety of peel, the following absorbances were noted as: 0.052, 0.122, 0.163, 0.193 and 0.232 respectively for the above five concentrations.

Table 2. Comparative efficacy on reducing power capacity of petroleum ether and methanol extract of different varieties of citrus fruit peel. The effects of 100 µg/mL peel extracts of different varieties of fruit were shown. The effects of ascorbic acid (control) on reducing power capacity were similarly done except giving peel extract. The results are means of ± standard deviation for three values in each group of sample.

Name of sample	Reducing power capacity (absorbance at 700 nm)	
	Petroleum ether (PE) fraction	Methanol (Me) fraction
Ascorbic acid	3.173 ± 0.001	3.170 ± 0.01
C. limittoids (So)	0.760 ± 0.01	1.556 ± 0.01
C. hystrix (Sat)	0.740 ± 0.01	0.015 ± 0.0005
C. medica (Ja)	2.200 ± 0.10	1.626 ± 0.0005
C. reticulate (Kh)	2.613 ± 0.01	2.740 ± 0.01
C. lemon (Le)	1.723 ± 0.005	0.868 ± 0.001

The experimental findings indicated that the total antioxidant capacity had enhanced dose dependently and the effects for *C. reticulate* (Kh) were much higher than the previous three varieties when compared to the control. Among the five varieties of peels, the higher potency on total antioxidant capacity was found for *C. reticulate* (Kh) (Fig. 1).

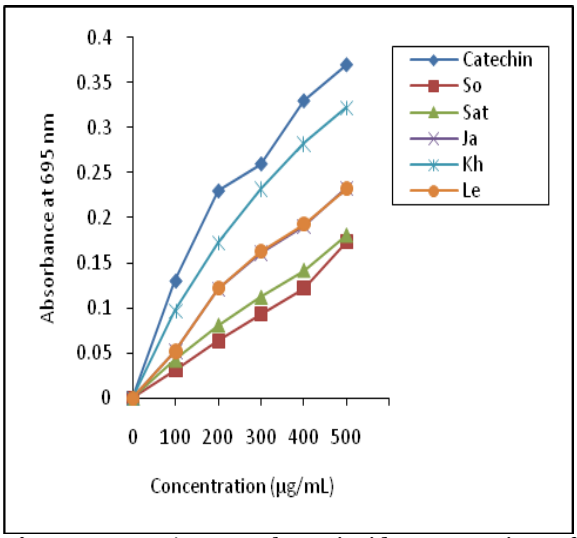

Fig. 1. *In vitro* total antioxidant capacity of petroleum ether extract of *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) varieties of citrus fruit peel. Different concentrations of peel extracts or catechin (100, 200, 300, 400 and 500 µg/mL) were used in the assay and the absorbances were recorded.

Therefore, the different peel extracts were essential constituents extracted from petroleum ether and showed antioxidative effects however; *C. reticulate* (Kh) *and C. lemon* (Le) varieties of peel exhibited the potent effects regarding this phenomenon.

Effects of different concentrations of peel extracts treated with methanol on total antioxidant capacity

To examine the effectiveness of purification of phytochemicals, petroleum ether and methanol extractions were performed in this study.

As shown in Fig. 2, total antioxidant capacity of five varieties of fruit peels of different concentrations were shown. For *C. limittoids* (So) variety, the absorbance of different doses of peel extracts (100, 200, 300, 400 and 500 µg/mL) were recorded as 0.052, 0.106, 0.129, 0.162 and 0.204 respectively while for *C. hystrix* (Sat) variety of peel, the absorbance 0.068, 0.146, 0.213, 0.250 and 0.320 respectively were found for the above concentrations. Similarly, the absorbances for C. medica (Ja) variety of peel were recorded as 0.037, 0.067, 0.097, 0.125 and 0.138 respectively for different concentrations of extracts. The results demonstrated that the total antioxidant capacity had increased gradually in presence of the increased concentrations. Among the different three varieties, the higher absorbances were recorded for *C. hystrix* (Sat) variety when compared to the control catechin however other two varieties of peel also showed potent antioxidant capacity when compared to the control.

The absorbance of catechin were found to 0.13, 0.23, 0.26, 0.33 and 0.37 respectively for different concentrations (100, 200, 300, 400 and 500 µg/mL) and increased dose dependently. Total antioxidant capacity were also examined for another two varieties, *C. reticulate* (Kh) and *C. lemon* (Le) where the

following absorbance were recorded for *C. reticulate* (Kh) for the similar doses of extracts of peels: 0.052, 0.090, 0.121, 0.151 and 0.177 respectively. Similarly, the following absorbances for *C. lemon* (Le) variety of peel were determined respectively for the above five different concentrations: 0.031, 0.048, 0.073, 0.091 and 0.120. The findings indicated that the total antioxidant capacity of the two species of peel had enhanced dose dependently, however the effects were much pronounced for *C. reticulate* (Kh) extract when compared to the control. *C. lemon* (Le) also showed potent antioxidant capacity because of the increasing absorbance although the values were lower than catechin. The petroleum ether extraction of two varieties of peel, *C. reticulate* (Kh) and *C. lemon* (Le) were assumed to be more effective than methanol extraction (Figure 1 and 2). Among the five varieties of peels during methanol extraction, the higher potency on total antioxidant capacity was found for *C. hystrix* (Sat) extract (Fig. 2).

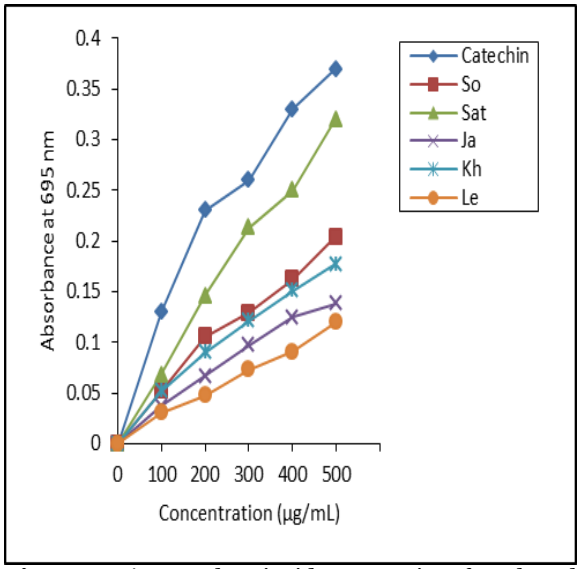

Fig. 2. *In vitro* total antioxidant capacity of methanol extract of *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) varieties of citrus fruit peel. Different concentrations of peel extracts or catechin (100, 200, 300, 400 and 500 µg/mL) were used in the assay and the absorbances were recorded.

Effects of different concentrations of peel extracts treated with petroleum ether on reducing power capacity

As shown in Fig. 3, the reducing power capacity of petroleum ether extract of citrus peel, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) has been demonstrated. For *C. limittoids* (So) variety, the absorbance of different concentrations (20, 40, 60, 80 and 100 µg/mL) were determined as 0.188, 0.42, 0.58, 0.64 and 0.76 respectively while for *C. hystrix* (Sat) variety, the values for the above mentioned concentrations were found as 0.25, 0.406, 0.52, 0.573 and 0.74 respectively. The absorbances of the standard ascorbic acid for the above concentrations were found as 2.032, 2.826, 3.016, 3.144 and 3.173 respectively. Similarly, the absorbances of the different concentrations (20, 40, 60, 80 and 100 µg/mL) of the extract of C. medica (Ja) were noted as 1.2, 1.52, 1.76, 1.94 and 2.2 respectively. Among the three varieties of peel, the higher absorbances were found for *C. medica* (Ja) against different concentrations however the values were lower when compared to the standard ascorbic acid. The other two varieties of peels also showed the potent reducing power capacity due to the increasing absorbance. Total reducing power capacity was enhanced for the above three varieties dose dependently. Similar increasing tendency was shown for the standard ascorbic acid at different concentrations (Fig. 3). The reducing power capacity of another two varieties of peel, *C. reticulate* (Kh) and *C. lemon* (Le) was also examined during petroleum ether extraction for different extract concentration (20, 40, 60, 80 and 100 µg/mL). The increased and pronounced reducing power capacity were demonstrated for *C. reticulate* (Kh) and *C. lemon* (Le) and the absorbance were increased dose dependently showing higher antioxidative effects of the peel. As shown in Fig. 3, *C. reticulate* (Kh) variety of peel showed higher potency on reducing power capacity (absorbance at 700 nm: 1.463, 1.97, 2.36, 2.463 and 2.613 respectively) against different doses of extract rather than *C. lemon* (Le) (absorbance at 700 nm: 0.78, 1.26, 1.453, 1.573 and 1.723 respectively). Therefore, all the varieties of peel extract produced the increased reducing power capacity when compared to standard ascorbic acid however; *C. reticulate and C. medica* showed the potential role on antioxidative effects.

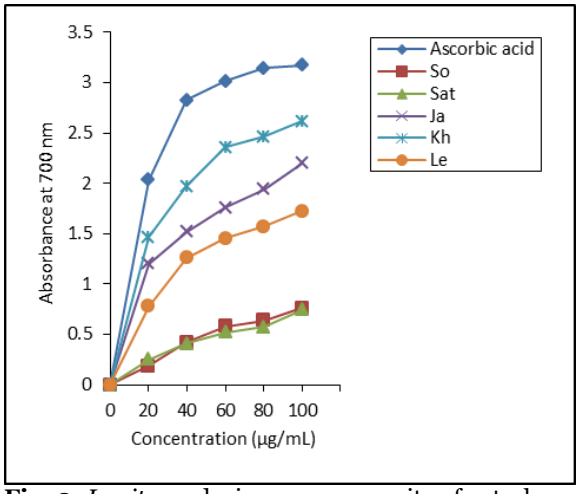

Fig. 3. *In vitro* reducing power capacity of petroleum ether extract of *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) varieties of citrus fruit peel. Different concentrations of peel extracts or ascorbic acid (20, 40, 60, 80 and 100 µg/mL) were used in the assay and the absorbances were recorded.

Effects of different concentrations of peel extracts treated with methanol on reducing power capacity

Fig. 4 shows the reducing power capacity of methanol extract of citrus peel varieties, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le). The absorbance were recorded in response to different concentrations of extracts of peel (20, 40, 60, 80 and 100 µg/mL) and compared to standard ascorbic acid for the similar concentrations. For *C. limittoids* (So), the absorbance in different concentrations (20, 40, 60, 80 and 100 µg/mL) were recorded as 0.168, 0.201, 0.45, 1.373 and 1.556 respectively and for *C. hystrix* (Sat), the values for the above mentioned concentrations were 0.472, 0.78, 0.863, 0.94 and 0.015 respectively obtained. The absorbance of different doses of extract was recorded similarly as 0.775, 1.136, 1.424, 1.563 and 1.626 respectively for *C. medica* (Ja) variety. The reducing power capacity was enhanced for the above three varieties dose dependently. The higher reducing power capacity for *C. medica* (Ja) was observed when compared to ascorbic acid however other varieties of extracts of traits also showed the potent reducing power capacity compared to standard ascorbic acid. All the three varieties of peel showed the higher potency on antioxidative effects and the effects were enhanced in response to the increasing concentrations although the values were lower than ascorbic acid. Total reducing power capacity was also examined from methanol treatment for another two varieties, *C. reticulate* (Kh) and *C. lemon* (Le) where the absorbance were found as 1.473, 2.133, 2.36, 2.63 and 2.74 respectively for *C. reticulate* (Kh) variety for the similar doses of extracts of peels. The values were increased for different extract concentrations respectively when compared to ascorbic acid. The respective absorbances for ascorbic acid were recorded as 2.026, 2.7, 3.015, 3.14 and 3.17 and the values were increased for increasing concentrations. The extract *C. lemon* (Le) also caused the higher potency and reducing power capacity (absorbance at 700 nm: 0.414, 0.613, 0.723, 0.82 and 0.868 respectively) during methanol extraction. Therefore, all these different varieties of fruits had the potent reducing power capacity in response to different extract concentrations and the results were appeared to indicate that *C. reticulate* and *C. medica* species of peel played the potential role regarding this phenomenon. Although petroleum ether extract of these species of peel showed the potent effects however methanol extracts cause much higher and pronounced results showing the higher antioxidative effects (Fig. 4 and 3). The peel extracts after methanol treatment produced higher specificity thereby assumed to be effective extraction strategy for the separation of phytochemicals.

Phytochemical screening and comparative analysis of fruit peels

In Table 1, the two fractions PE (petroleum ether) and Me (methanol) have been shown and their effects on antioxidative activity were compared and evaluated. The effects were demonstrated for maximal concentration of peel extracts (500 µg/mL). Among the petroleum ether fractions, *C. reticulate* (Kh), *C. medica* (Ja) and *C. lemon (*Le*)* extract showed higher potency on antioxidative effects when compared to the standard catechin. The other two varieties, *C. hystrix* (Sat) and *C. limittoids* (So) (PE fraction) although produced potent effects however the values were much lower than the standard catechin.

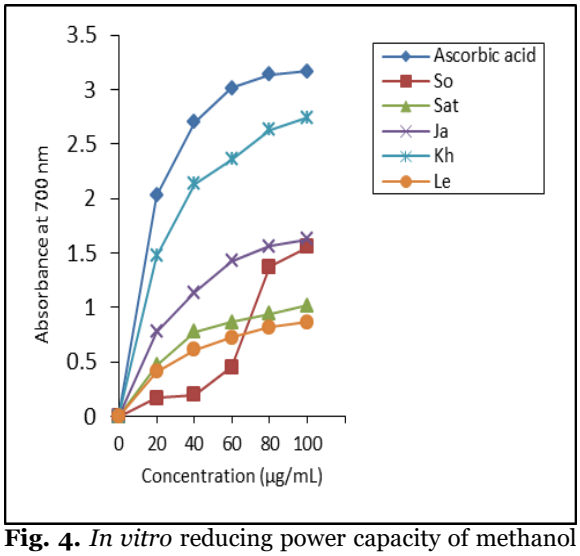

Fig. 4. *In vitro* reducing power capacity of methanol extract of *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) varieties of citrus fruit peel. Different concentrations of peel extracts or ascorbic acid (20, 40, 60, 80 and 100 µg/mL) were used in the assay and the absorbances were recorded.

As illustrated in Table 1, the higher antioxidative activity for the varieties, *C. hystrix* (Sat) and *C. limittoids* (So) after methanol treatment (Me fraction) were observed when compared to catechin. Although the absorbance values against standard catechin were lower, the extracts showed much higher potency on antioxidative effects because of the increasing absorbance demonstrating that the methanol extract of the fruit peel may have some potential compounds responsible for the prevention of complications caused by the microorganisms. The highest activity was observed in the extract of *C. reticulate* (Kh) (PE fraction) for the maximal concentration of peel extract (500 µg/mL) when compared to the same species in methanol extraction (absorbance at 500 nm: *C. reticulate* (Kh) 0.322 (PE) and 0.177 (Me). The results are appeared to indicate that some species of fruit peel exhibited higher potency on antioxidative effects. Similar trends of antioxidative effects were demonstrated for *C. medica* (Ja) and *C. lemon (*Le*)* extract during preparations in petroleum ether extractions when compared to control while methanol fractions for this species of peel show lower potency on antioxidative effects (absorbance at 569: 0.233 and 0.232 (PE fraction); 0.138 and 0.120 (Me fraction). Among the PE fractions, *C. limittoids* (So) showed much less absorbance values when compared to the effects of standard catechin. Similar results were noted for *C. hystrix* (Sat) species (methanol fractions) showing much increased antioxidative effects when compared to katechin and the effects were much lower for petroleum ether fractions (shown in Fig. 3 and Table 1). Among the Me fractions, *C. lemon* (Le) showed much less absorbance values when compared to the control. Therefore, it is obvious that methanol extracts of the species may have some essential constituents demonstrating the higher antioxidative effects. Therefore, the results are good agreement that both these fractions have antioxidative effects however the values of absorbance were higher than those of methanol fractions. As shown in Table 2, the reducing power capacity of different species of fruit peel, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) for petroleum ether (PE) and methanol (Me) fractions have been shown and the effects were demonstrated for maximal concentrations of peel extracts (100 µg/mL). Among the petroleum ether fractions, *C. reticulate* (Kh), *C. medica* (Ja) and *C. lemon (*Le*)* extract showed higher potency on reducing power capacity when compared to the standard ascorbic acid. The other two varieties, *C. limittoids* (So) and *C. hystrix* (Sat) also produced potent effects because of their increasing absorbance however the values were much lower than the standard ascorbic acid.

On the contrary, the reducing power capacity for Kh (*C. reticulate*), Ja (*C. medica*) and Sat (*C. hystrix*) in methanol extraction had much higher potency when compared to ascorbic acid. While the other two varieties, Sat and Le caused much lower effects when compared to control although the extracts were effective because of their increasing concentrations. The maximal activity was observed in the extract of *C. reticulate* (Kh) (Me fraction) for the maximal concentration of peel extract (100 µg/mL) when compared to the same species in petroleum ether extraction (absorbance at 700 nm: *C. reticulate* 2.613, PE fraction and 2.74, me fraction).

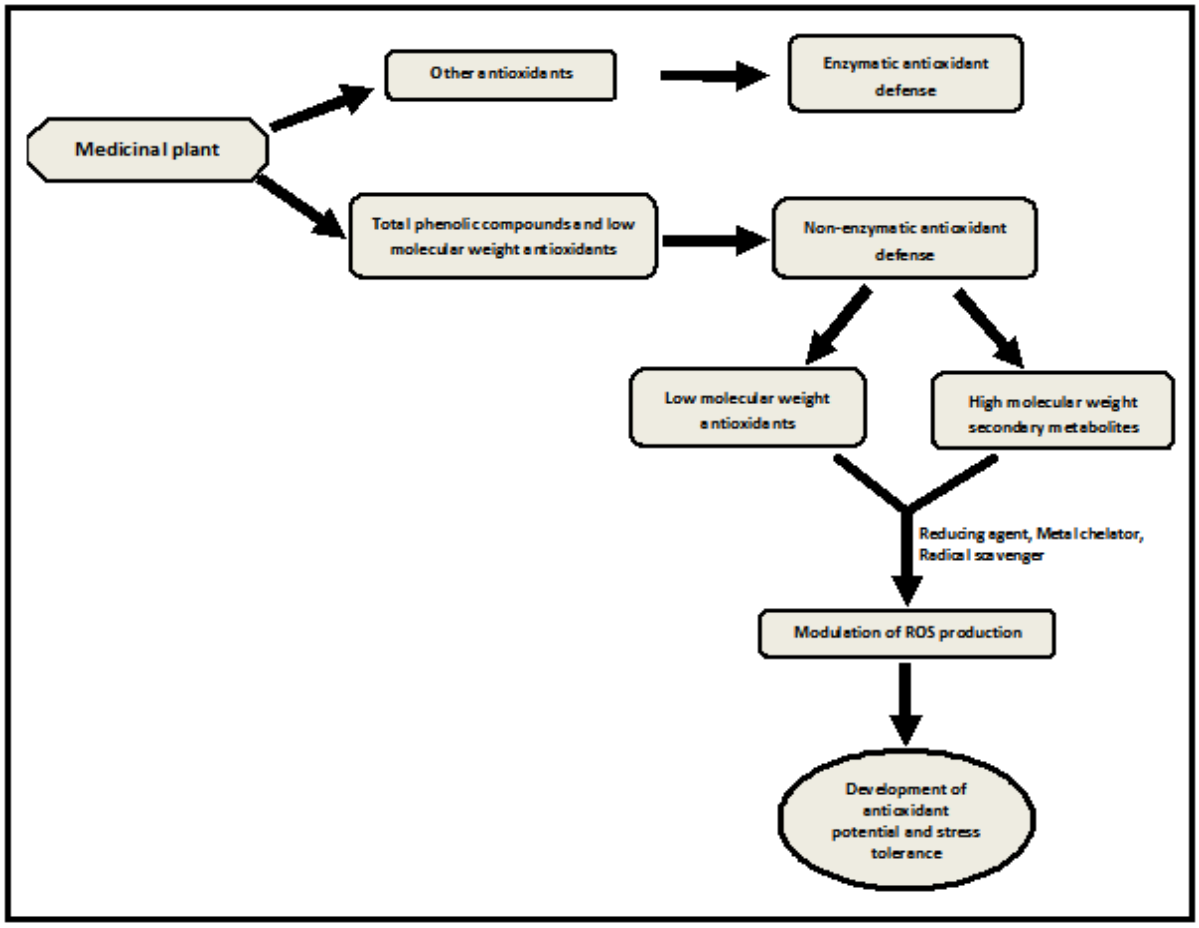

Fig. 5. Mechanism regarding the beneficial aspects of medicinal plant and development of antioxidant potential in response to environmental stimuli. Under biotic and abiotic stress conditions, the production of reactive oxygen species (ROS) increases resulting in induction of oxidative stress. Other antioxidants include superoxide dismutase (SOD), ascorbate peroxidase, glutathione S transferase, catalase (CAT) and peroxidase destroy toxic peroxides during increased oxidative stress through enzymatic antioxidant defense, while total phenolic compounds and low molecular weight antioxidants from plants cause the non-enzymatic antioxidant defense. Administration of several low molecular weight antioxidants (e.g., vitamins A, E, C, carotenes) and high molecular weight secondary metabolites such as tannins, flavonoids etc. extracted from plants, causes the generation of non-enzymatic antioxidant defense by functioning as free radical scavengers, reducing agents and metal chelators thereby augmenting the development of antioxidant potential and stress tolerance.

The results are appeared to indicate that some species of fruit peel exhibited higher potency on antioxidative effects. Similar trends of reducing power capacity were demonstrated for *C. medica* (Ja) and *C. lemon (*Le*)* during preparations in petroleum ether extractions when compared to control while methanol fractions for these species of peel showed lower potency on antioxidative effects respectively (absorbance at 700 nm: 2.2 and 1.723 for PE fraction); 1.626 and 0.868 for Me fraction). It was observed that the higher antioxidant capacity in petroleum ether extract of citrus peel was given by *C. reticulate* (Kh), *C. medica* (Ja) *and C. lemon* (Le) peel against the standard catechin (Fig. 1, Table 1). On the other hand, the peels of *C. hystrix* (Sat) *and C. limittoids* (So) showed lower antioxidant capacity (Table 1). The trend of overall antioxidative effects of petroleum ether extract of citrus peels according to their absorbance is given bellow:

Kh > Ja > Le > Sat > So

Among the methanol extract of citrus peel, the higher antioxidant capacity was observed by *C. hystrix* (Sat) and *C. limittoids* (So) peel when compared *to* the

standard catechin (Fig. 2, Table 1) and the lower effects were given by the peel of *C. reticulate* (Kh), *C. medica* (Ja) and *C. lemon* (Le) against the maximal response of peel extract (500 µg/mL) (Table 1). The trend of overall antioxidative effects of methanol extract of citrus peel according to their absorbance is given bellow:

Sat > So > Kh > Ja > Le

It was observed that the higher reducing power capacity in petroleum ether extract of citrus peel was given by *C. reticulate* (Kh), *C. medica* (Ja) and *C. lemon* (Le) peel against the standard ascorbic acid (Fig. 3, Table 2). On the other hand, the peels of *C. limittoids* (So) *and C. hystrix* (Sat) showed lower reducing power capacity (Fig. 3, Table 2). The trend of overall reducing power capacity of petroleum ether extract of citrus peel according to their absorbance is given bellow:

Kh > Ja > Le > So > Sat

Among the methanol extract of citrus peel, the higher reducing power capacity were recorded by *C. reticulate* (Kh), *C. medica* (Ja) *and C. limittoids* (So) peel when compared to the standard ascorbic acid (Fig. 4, Table 2) and the lower absorbance were given by the peel of *C. lemon* (Le) and *C. hystrix* (Sat) (Fig. 4 and Table 2). The trend of overall reducing power capacity of methanol extract of citrus peel according to their absorbance is given bellow:

Kh > Ja > So > Le > Sat

Discussion

Citrus fruit peel varieties and its different antioxidative effects

The comparative efficacy and evaluation on total antioxidant capacity and reducing power capacity of five citrus fruit peel varieties, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) have been demonstrated in the current study. Fruit peels are the major sources of phytochemicals and have been recognized to be involved in prevention of diverse complications caused by microorganisms. Citrus fruits are rich sources of useful phytochemicals such as vitamins A, C and E, mineral elements, flavonoids, coumarins, limonoids, carotenoids, pectins and other compounds (Zou *et al*., 2016). For the prevention of oxidative stress induced by environmental stimuli either biotic or abiotic, phytochemicals play the critical role because of their potent antioxidative effects. The current investigation regarding this phenomenon therefore has been undertaken and different fruit peels have been shown to be involved to exert their potential antioxidative effects. These phytochemicals consumed through fresh fruits or their derived products, have been suggested to have a wide variety of biological functions including antioxidant, antiinflammation, antimutagenicity, anticarcinogenicity and anti-aging to human health (Rajendran *et al*., 2014; Ke *et al*., 2015). Therefore, it is generally accepted and reasonable that extraction of phytochemicals from the fruit peel is of great importance in the prevention of diverse clinical complications.

Total antioxidant capacity in citrus peels treated with petroleum ether and methanol

Although different approaches and strategies have been employed to identify the compounds regarding this phenomenon, however in the current study the extractions were carried out by petroleum ether and methanol as potent organic solvents. In response to different concentrations of extract (100, 200, 300, 400 and 500 µg/mL) of the different varieties of fruit peel, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le), the total antioxidant capacity were increased dose dependently showing that the petroleum ether extract of peels were very active fractions during separation of the compounds. Among the different varieties of peel, the total antioxidant capacity was much higher for *C. reticulate* (Kh), *C. medica* (Ja) and *C. lemon* (Le) however for other varieties of peel (Sat and So), the absorbance values were comparatively lower although the peels were very active ingredient because of their increasing absorbance. Therefore, it is assumed that petroleum ether causes extractions of some essential compounds from the peels and thereby is recognized to be as a potent organic solvent for purification of the

compounds having antioxidative effects. It has been demonstrated from the previous investigations that phytochemicals were extracted through petroleum ether and were found to play the role on antioxidative effects (Biswas *et al.*, 2010; Hossain *et al.*, 2018). Singh *et al.* (2008) examined the antioxidant activity and found that the free radical scavenging activity of the different fractions of petroleum ether extract of *P. nigrum* (PEPN) had increased in a concentration dependent manner. Their findings are compatible to the current findings and have been shown to support strongly the result. It has been revealed from the previous studies that the antioxidant activity of plant extracts is strongly dependent on the solvent due to the different antioxidant potentials of compounds with different polarity (Upadhyay *et al.*, 2013; Singh *et al.*, 2014). Similarly, methanol extraction also causes the findings of antioxidant capacity of different fruit peels and the extract of different concentrations (100, 200, 300, 400 and 500 µg/mL) of different peels, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) shows the higher absorbance and the absorbance were found to increase dose dependently. Therefore, methanol extract is also very active fraction and may have some potent compounds showing antioxidant capacity. Among the different varieties of peels, *C. hystrix* (Sat) and *C. limittoids* (So) were shown to have higher absorbance demonstrating the higher antioxidant capacity. Although the absorbance were lower when compared to catechin, the peel extract of *C. reticulate* (Kh), *C. medica* (Ja) and *C. lemon* (Le) were very active and potent during preparation with methanol and show antioxidative effects since their absorbance were increased similarly dose dependently.

The higher antioxidant capacity and antioxidative effects in fruits were observed after methanol extractions as demonstrated by the previous investigation (Jan *et al.*, 2013). The results are very compatible and strongly supported by their findings. Therefore, both petroleum ether and methanol are assumed to be very potent organic solvents and cause the extractions of compounds from the peels. Although not identified, it is assumed that some phytochemicals are present in the fruit peels and show antioxidative effects. Several lines of evidences are pointed to suggest that fruit peels are very active ingredients and cause antioxidative effects (Mehra *et al.,* 2015; Abudayeh *et al.*, 2019).

Implication of antioxidative effects on clinical importance

Citrus fruit is popular due to its characteristic flavor, taste, aroma and numerous health benefits. Processing of citrus fruits into different products or their consumption as such produce by-products such as peel, seed and pulp which are usually wasted. This waste contains various bioactive compounds such as ascorbic acid, phenolic compounds etc as demonstrated by Ullah *et al.* (2014). Moreover, previous studies reported that the peel of pomelo fruit had contained a higher amount of antioxidant content and antioxidant capacity as compared to its pulp (Toh *et al.*, 2013). More importantly, the prevention of many chronic diseases, such as cancer, diabetes and cardiovascular disease, has been suggested to be associated with the antioxidant activity (Rajendran *et al.*, 2014). Therefore, a deep study of natural antioxidants, such as those from fruits and vegetables, is of great importance to human health.

Plants are the major source of natural antioxidants due to the presence of various biophenolic compounds like phenolic acids, saponins, flavonoids and tocopherols (Abbas *et al.*, 2015). Plant materials which are rich in phenol contents, are widely used as medicinal remedies due to their various pharmacological properties (Abbas *et al.*, 2015).

Flavonoids are naturally-occurring compounds of plants and account for different phenolic compounds. They have been shown to effectively scavenge most oxidizing molecules, which include singlet oxygen and other free radicals (Treml and Šmejkal, 2016). The antioxidative effects observed in different varieties of fruit peels in the current study are therefore, very important findings in the field of elucidation of phytochemicals as demonstrated by the above several lines of investigations.

Reducing power capacity in citrus peels treated with petroleum ether and methanol

Reducing power capacity was determined from petroleum ether and methanol fractions of the different fruit peels, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le). The absorbance of different concentrations of peel extract (20, 40, 60, 80 and 100 µg/mL) was increased dose dependently showing the higher antioxidative effects of the fruit peels. For the different peel species, *C. reticulate* (Kh), *C. medica* (Ja) and *C. lemon* (Le) showed higher effectivity on reducing power capacity and thereby higher antioxidative effects were demonstrated when purified through petroleum ether extraction. Although the effects of other two varieties of peels (So and Sat) were lower when compared to ascorbic acid, however they were also considered to be potent varieties of fruit peel and showed antioxidative effects because of their increasing absorbance against ascorbic acid. Therefore, all the varieties of fruit peel are biologically active and may have some essential bioactive compounds. Several lines of evidences are strongly suggested in this connection that foods containing phytochemicals, such as fruits and vegetables containing antioxidants, have protective effects against disease. It has been revealed from the previous study that the reducing power capacity were determined from the biological sample extracted with petroleum ether and were found to be enhanced (Biswas *et al.*, 2010; Mukhija and Kalia 2014). Moreover, the absorbance of all extracts and standard is a function of their concentrations, and generally, increases linearly with the increase in concentration as shown by some researchers (Islam *et al.*, 2018).

The results recorded after petroleum ether treatment in the current study, are supported by their findings as the reducing power capacity in the extract were enhanced in their experiment. Similarly, methanol extractions of different fruit peels were examined to find the reducing power capacity and the absorbance of peel extracts of different concentrations (20, 40, 60, 80 and 100 µg/mL) were enhanced dose dependently showing the peels were very active and exhibited antioxidative effects. Among the different varieties of peels, *C. reticulate* (Kh), *C. medica* (Ja) and *C. limittoids* (So) were shown to have higher reducing power capacity and played the potential role however other varieties of peel (Sat and Le) also showed potent antioxidative effects although the values were comparatively lower against the standard ascorbic acid. Therefore, both petroleum ether and methanol extracts of peels were found to have diverse antioxidative effects and may have some essential bioactive compounds. It has been demonstrated from the previous observations that antioxidative effects were enhanced in response to the extracts of peel through methanol solvent extraction (Chanda *et al.*, 2011; Hossain *et al.*, 2018). Dar *et al.* (2014) demonstrated that the methanolic root extract of *Mentha arvensis* L. had showed good reducing power when compared to the standard ascorbic acid.

The results are compatible to their findings and are strongly supported. The separation of the compounds from the fruit peels with petroleum ether and methanol are therefore, very essential extraction strategy causing the purification of phytochemicals and thereby the higher antioxidative effects were observed.

Role of antioxidative effects on the prevention of oxidative stress

The increased antioxidant capacity and reducing power capacity of peel varieties are of great importance in biological system. In adverse environmental circumstances, reactive oxygen species (ROS) are produced thereby metabolic alterations and cellular impairment are observed however phytochemicals present in the plants play the pivotal role because of their antioxidative effects. Excess production of ROS in response to biotic and abiotic stresses has been shown to cause oxidative stress leading to cellular damage and ultimately cell death. To prevent or alleviate the ROS induced damage allowing the beneficial functions of ROS to continue, plants have evolved an intriguing antioxidant defence system which functions to keep levels of reactive or active oxygen species under control. Antioxidant

defence systems comprise both enzymatic as well as non-enzymatic components. The production of ROS in response to environmental stress is reduced through these defense systems and thereby the stress tolerance is observed. Phytochemicals are major ingredients available in plant kingdom and are considered to be involved in the prevention of oxidative stress caused by both biotic and abiotic stress in the environment. These environmental stimuli affect the living organisms and cause metabolic alterations however, because of the presence of antioxidative effects, phytochemicals in fruits or fruit peels were found to neutralize the effects. Admininistration of phytochemicals extracted from plants has been shown to exert their effects and antioxidant potential is developed as shown in Fig. 5. The ROS is very powerful chemicals causing diverse biochemical and biological adverse effect in the organisms. Phytochemicals present in the plant kingdom plays the critical role through the antioxidative functions. The enhanced antioxidative effects in the fruit peels in the present study might be due to the presence of some essential phytochemicals. It is assumed that fruit peel having active ingredients and causes antioxidative effects in the biological system and the proposed mechanism of the antioxidative functions are illustrated in Fig. 5. It is believed that they scavenge radicals by inhibiting initiation and breaking of chain reaction, suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide and quenching superoxide and singlet oxygen. The protective action of fruits and vegetables has been attributed to the presence of antioxidants, especially anti-oxidant vitamins including ascorbic acid, a-tocopherol and b-carotene (Pertuzatti *et al.*, 2014). However, the previous study has conclusively shown that the majority of the anti-oxidant activity may be from compounds such as flavonoids, isoflavone, flavones, anthocyanin, catechin and isocatechin rather than from Vitamin C, E and β-carotene (Wang *et al.*, 1996). Epidemiological studies have shown that consumption of food and beverages rich in phenolic content can reduce the risk of heart disease by slowing the progression of atherosclerosis by acting as anti-oxidants towards low-density lipoprotein (LDL) (Frankel *et al.*, 1995). Therefore, mostly, the current focus is on the anti-oxidant action of phenolics. The anti-oxidant activity of phenolics is mainly because of their redox properties which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Kasote *et al.*, 2015). Collectively, citrus peels of different varieties are very effective showing both total antioxidant capacity and reducing power capacity and might be involved in causing the development of antioxidative potential to the living organisms so that they survive in the environment. Our findings are useful and good agreement for the evaluation of antioxidative effects of peel extract in petroleum ether and methanol augmenting the elucidation of phytochemicals and may give an insight for development of the separation techniques of these phytochemicals.

Conclusion

Environment is the major stimulus affecting metabolic alterations. The environmental stimuli particularly biotic and abiotic are believed to cause oxidative stress which is harmful to the organisms and causes metabolic impairment and produces reactive oxygen species (ROS). These ROS species cause cell damage and other harmful effects to the biological system. Therefore, it is substantial to prevent the oxidative stress causing the adverse environmental stimuli. Phytochemicals are essential compounds involved in prevention of oxidative stress and are widely distributed to the plant organisms. These bioactive compounds are located abundantly to the citrus fruits which play the critical role regarding this matter. In this study, different five varieties of fruits peels, *C. limittoids* (So), *C. hystrix* (Sat), *C. medica* (Ja), *C. reticulate* (Kh) and *C. lemon* (Le) were examined and the antioxidative effects particularly total antioxidant capacity and reducing power capacity were assayed and evaluated through organic solvent extraction. Although different varieties of citrus peel showed the efficacy regarding the antioxidative effects in our study, however, the effects were separated on the basis of their purification pattern. Among the five different

varieties of species of peel, both petroleum ether and methanol fraction show the potent antioxidative effects however, methanol extractions were found to be involved in playing the vital role on antioxidative effects in different peel species. Among the five different varieties of peel, *C. reticulate* and *C. medica* species were assumed to be potential on antioxidative effects showing the presence of some phytochemicals which are effective on prevention of oxidative stress caused by microorganisms or other environmental stresses.

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