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STUDIES OF PRELIMINARY PHYTOCHEMICAL SCREENING, MEMBRANE STABILIZING ACTIVITY, THROMBOLYTIC ACTIVITY AND *IN-VITRO* ANTIOXIDANT ACTIVITY OF LEAF EXTRACT OF *CITRUS HYSTRIX*

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ABSTRACT: In this present study, the leaf extracts of *Citrus hystrix* were subjected to a comparative evaluation of preliminary phytochemical screening, membrane stabilizing activity, thrombolytic activity and *In-vitro* antioxidant activity. The thrombolytic and membrane stabilizing activities were assessed by using human erythrocyte and the results were compared with standard streptokinase (SK) and standard anti-inflammatory drug, acetyl salicylic acid (ASA), respectively. For thrombolytic activity and membrane stabilizing activity ethanolic leaf extract exhibited clot-lysis value of 13.69% against standard SK (37.43 %) and highest percentage of hemolytic value of 74.40% against standard ASA (93.24%) respectively. DPPH free radical scavenging activity, Cupric Reducing Antioxidant Capacity (CUPRAC), Total Phenolics Content, Total Flavonoids Content, Nitric oxide scavenging assay and total antioxidant capacity were done in the present study. In case of *in-vitro* antioxidant evaluation all extract forms showed a significant positive results. Phytochemical investigation suggested the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenols, steroids and tannins.

INTRODUCTION: *Citrus hystrix* (Family-Rutaceae) is locally known as Satkora. English name is Kaffir lime. *Citrus hystrix* tree is small and shrubby with aromatic and distinctive "double" shaped leaves which are evergreen, *Citrus* hystrix typically grows 3 to 6 m tall. The globose flower buds open into fragrant flowers with 4 to 5 petals and around 30 stamens; petals are white with reddish or pink on the outside.

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The leaves can be rubbed on to gums and teeth for total dental health. The oil is a great hair and scalp cleanser. The oil from the rind has strong insecticidal properties. *Citrus hystrix* native to Indochinese and Malaysian eco regions in India, Nepal, Philippines, Bangladesh, Indonesia, Malaysia and Thailand [1]. In Bangladesh, both green and mature fruits are used for cooking and pickle preparation.

The sweet flavor of fruits due to essential oil of flavenoid parts may be used in flavoring purposes in perfume industries. Every year, Bangladesh earns handsome amount of foreign currency by exporting these fruits especially to UK, USA and Middle East countries. Like other *citrus* species Sat Kara is also propagated conventionally by means of

seeds, grafting and budding methods [2]. As a part of our continuing studies on medicinal plants of Bangladesh the organic soluble materials of the leaf extracts of *Citrus hystrix* were evaluated for phytochemical screenings, anti-thrombolytic activity, membrane stabilizing and *in-vitro* antioxidant activity for the first time[3-10].

MATERIALS AND METHODS:

Collection and Processing of Plant Samples: Plant sample of *Citrus hystrix* was collected from Jayantapur, Sylhet, Bangladesh in November, 2014 and a plant sample was submitted to the Bangladesh National Herbarium for identification (Accession number 38758**).** Leaf was sun dried for seven days. The dried leaf were then ground in coarse powder using high capacity grinding machine which was then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

Extraction Procedure:

The powdered plant parts (22 gm) were successively extracted in a soxhlet extractor at elevated temperature using 250 ml of distilled Methanol (40-60)°C which was followed by ethanol, and chloroform. After extraction all extracts kept in refrigerator 4°C for future investigation with their necessary markings for identification.

Photochemical Screening: Different extracts were screened for the presence of phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterols and carbohydrate by using standard protocols [11].

Streptokinase (SK):

Commercially available lyophilized altepase (Streptokinase) vial (Popular pharmaceutical Ltd.) of 15, 00,000 I.U, was collected and 5ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for in vitro thrombolytic activity evaluation.

Blood sample:

Blood (n=5) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1ml of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

Thrombolytic activity: The thrombolytic activity of all extracts of the plants was evaluated by the method developed by Prasad *et al*., 2006 using, streptokinase (SK) as the standard [12-14].

Membrane stabilizing activity: The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane[15]. The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced [16]. To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes containing anticoagulant EDTA (3.1% Na- Citrate). The blood was centrifuged and blood cells were washed three times with solution (154mM NaCl) in 10mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g.

Hypotonic solution- induced haemolysis*:* The test sample consisted of stock erythrocyte (RBC) suspension (0.50mL) mixed with 5mL of hypotonic solution (50mM NaCl) in 10mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (1.0mg/mL) or acetyl salicylic acid (0.1mg/mL). The control sample consisted of 0.5mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10min at 3000g and the absorbance of the supernatant was measured at 540nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation- % inhibition of haemolysis = 100 x (OD1-OD2/OD1).

Free Radical Scavenging Assay:

The free radical scavenging capacity of the extracts was determined using DPPH [17]. 1ml of plant extract or standard of different diluted (6.25µg/ml to 800µg/ml) concentration solutions was taken in test tube and freshly prepared 2ml of 0.004% DPPH solution is added in each test tubes to make the final volume 3ml. Incubate the mixture in room temperature for 30minutes, the absorbance was read at 517nm using a spectrophotometer. Ascorbic

acid was used as standard. Control sample was prepared containing the same volume without any extract and standard. The absorbance was read at 517nm using a spectrophotometer. Ethanol was served as blank. % inhibition of the DPPH free radical was measured by using the following equation:

% inhibition = $(1-A_1/A_0) \times 100\%$ …. [Equation 1]

where,

A_1 = Absorbance of the extract or standard

A_0 = Absorbance of the control

Cupric Reducing Antioxidant Capacity (CUPRAC):

The assay was conducted as described previously [18]. To 0.5ml of plant extract or standard of different diluted (5µg/ml to 200µg/ml) concentrations solutions was taken in test tube and added 1ml of copper (II) chloride solution (0.01 M prepared from $CuCl_2.2H_2O$), 1ml of ammonium acetate buffer at pH 7.0, 1ml of neocaproin solution (0.0075M) were mixed. The final volume of the mixture was adjusted to 4.1ml by adding 0.6 ml of distilled water and the total mixture was incubated for 1hour at room temperature. Then the absorbance of the solution was measured at 45nm using a spectrophotometer against blank. Ascorbic acid was used as a standard.

Determination of Total Phenolics Content:

Total phenolic contents in the extracts were determined by the Folin-Ciocalteu reagent method described by and [18-19]. All of extracts and standard were diluted by serial dilutions as (6.25µg/ml to 200 µg/ml) then, on each test tube containing 1ml of diluted solution of sample and standard, following reagent solutions were added 5 ml folin-ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml (7.5% sodium carbonate) of sodium carbonate. Samples were incubated at 20^0C temperature for 60 minutes and standard diluted solution–reagent mixture was incubated at 20^0C temperature for 30 minutes.

Absorbance of samples and standard were measured at 765 nm using spectrophotometer against blank. A typical blank solution contained the solvent used to dissolve the plant extract. The total content of phenol compounds in plant extracts in Gallic acid equivalents (GAE) was calculated using the following equation:

C = (c x V)/m …..... [Equation 2]

where; C = total content of phenol compounds, mg/gm plant extract, in GAE, c = the concentration of Gallic acid established from the calibration curve (mg/ml), V = the volume of extract in ml, m = the weight of crude plant extract in gm.

Determination of Total Flavonoids Content:

Aluminum chloride colorimetric method was used for flavonoids determination [20]. To 1 ml of plant extract or standard of different diluted (6.25µg/ml to 200µg/ml) concentrations solutions was taken in a test tube and added 3ml of methanol, 0.2ml of aluminum chloride, 0.2 ml of 1M potassium acetate and 5.6ml of distilled water. It incubated at room temperature for 30min then absorbance of the reaction mixture was measured at 415nm with spectrophotometer against blank. Methanol served as blank. The total content of flavonoid compounds in plant methanol extracts in quercetin equivalents was calculated with equation 2.

Nitric oxide Scavenging Assay:

Nitric oxide scavenging assay was carried by using sodium nitroprussid [21]. This can be determined by the use of the Griess Illosvoy reaction. 2ml of 10mM sodium nitroprusside in 0.5ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract/sub- fraction of different diluted (6.25µg/ml to 100µg/ml) concentrations solution and the mixture was incubated at 25°C for 150 minutes. From the mixture 0.5 ml was taken out and added into 1.0 ml sulphanilamide solution (0.33% in 20% glacial acetic acid) and further incubated at room temperature for 5 minutes. Finally, 1.0 ml Naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and maintained at room temperature for 30 minutes. The absorbance was measured at 546 nm. Typical control solutions contain the same solution mixture without plant extract or standard. % inhibition was calculated by using equation 1.

Determination of Total Antioxidant Capacity:

The total antioxidant capacity was evaluated by the phosphomolybdenum method [22]. 0.3ml of extract and sub-fraction in methanol, ascorbic acid used as standard (12.5-200µg/ml) and blank (methanol)

were combined with 3ml of reagent mixture separately and incubated at 95°C for 90 minutes. After cooling to room temperature, the absorbance of each sample was measured at 695nm against the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following equation:

A = (c x V)/m…….. [Equation 3]

Where, A = total content of antioxidant compounds, mg/gm plant extract, in ascorbic acid equivalent c = the concentration of ascorbic acid established from the calibration curve, mg/ml, V = the volume of extract in ml, m = the weight of crude plant extract, gm.

RESULTS:

Phytochemical Screening: The results of the various qualitative chemical tests for the detection of chemical constituents of the extract are placed in **Table 1**.

TABLE 1: RESULT OF CHEMICAL GROUP TEST OF LEAF EXTRACTS OF *CITRUS HYSTRIX*

Name of tests	Result
Carbohydrates	+
Glycosides	+
Alkaloids	+
Saponins	-
Flavonoids	+
Steroids	+
Tannins	+

"+" Present, "-'' Absent

In-Vitro* Thrombolytic Effect of *Citrus hystrix: As a part of discovery of cardio protective drugs from natural sources, different extract of *Citrus hystrix* were assessed for thrombolytic activity and the results are present in **Table 2**.

TABLE 2: CLOT LYSIS % BY DIFFERENT EXTRACTS OF *CITRUS HYSTRIX*

Samples	% of Clot Lysis
Methanol Extract	12.33 ± 0.04
Ethanol Extract	13.69 ± 0.06
Chloroform Extract	12.19 ± 0.05
Control	3.37 ± 0.01
Streptokinase	37.43 ± 0.10

± S.D (standard deviation)

Membrane stabilizing Activity:

The different extractives of *Citrus hystrix* at concentration 1.0mg/mL significantly protected the lysis of human erythrocyte membrane by hypotonic solution induced haemolysis compared to the standard acetyl salicylic acid (0.10mg/ml) **Table 3**.

TABLE 3: EFFECT OF EXTRACTIVES OF *CITRUS HYSTRIX* ON HYPOTONIC SOLUTION INDUCED HEMOLYSIS OF ERYTHROCYTE MEMBRANE

Samples	Concentration (mg/ml)	Hypotonic solution induced Haemolysis inhibition%
Acetyl salicylic acid	0.1	93.24±0.01
Methanol Extract	1	58.35±0.04
Ethanol Extract	1	74.40±0.05
Chloroform Extract	1	64.19±0.01

±S.D (standard deviation)

DPPH free radical scavenging assay: Three extracts exhibited considerable DPPH free radical scavenging activity as indicated by their values and this can be shown in **Fig.1**.

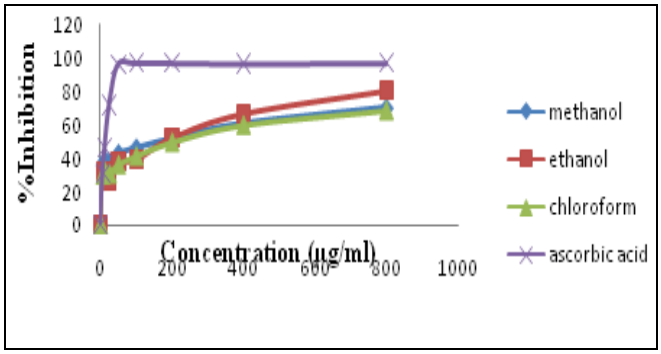

FIG.1: COMPARATIVE DPPH RADICAL SCAVENGING ACTIVITY OF EXTRACT AND ASCORBIC ACID

Cupric Reducing Antioxidant Capacity (CUPRAC): Reduction of Cu_2^+ ion to Cu^+ was found to rise with increasing concentrations of the different extracts. The standard ascorbic acid showed highest reducing capacity. Among the methanolic extracts of *Citrus hystrix* showed maximum reducing capacity that is comparable to ascorbic acid **Fig.2**.

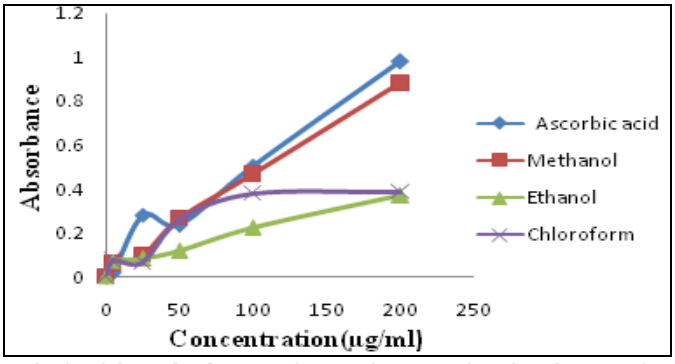

FIG. 2: COMPARATIVE CUPRIC REDUCING POWER OF EXTRACTS AND ASCORBIC ACID

Total Phenolic Content: Total phenolic content of the different extracts of was determined by using the Folin-Ciocalteu reagent and were expressed as Gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of Gallic acid. Methanol extract of was found to contain the highest amount of phenols **Table 4**.

TABLE 4: TOTAL PHENOL CONTENTS OF THE DIFFERENT EXTRACTS OF *CITRUS HYSTRIX*

Extract(s)	Total phenolic Contents (mg/gm), Gallic Acid Equivalent
Methanol	1.40 ± 0.32
Ethanol	1.37 ± 0.32
Chloroform	1.17 ± 0.05

±S.D (standard deviation)

Total Flavonoid Content: Aluminum chloride colorimetric method was used to determine the total flavenoid contents of the different extracts of *citrus hystrix*. Total flavonoid contents were calculated using the standard curve of quercetin and was expressed as quercetin equivalents (QE) per gram of the plant extract. Methanol extract of *citrus hystrix* was found to contain the highest amount of flavonoid **Table 5.**

TABLE 5: TOTAL FLAVONOID CONTENTS OF THE DIFFERENT EXTRACTS OF *CITRUS HYSTRIX*

Extract(s)	Total Flavonoid Contents (mg/gm, Quercetin Equivalent)
Methanol	2.58 ± 0.71
Ethanol	2.3 ± 0.56
Chloroform	2.33 ± 0.49

±S.D (standard deviation)

Nitric Oxide Scavenging Assay: Nitric oxide (NO) is very unstable species and reacting with oxygen molecule produce stable nitrate and nitrite which can be estimated by using griess reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease which can be measured at 546nm. Chloroform extract of *Citrus hystrix* leaf has potent nitric oxide scavenging activity (IC_{50} value 93.7µg/ml) **Table 6**. The scavenging of NO by the extract was increased in dose dependent manner. **Fig.3** illustrate a significant decrease in the NO radical due to the scavenging ability of same concentration extract and ascorbic acid

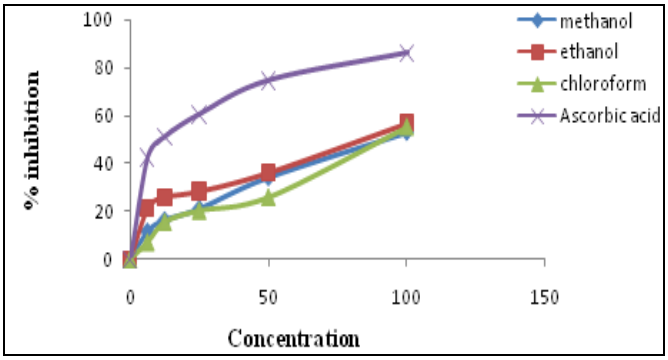

FIG. 3: COMPARATIVE NITRIC OXIDE SCAVENGING ACTIVITY OF *CITRUS HYSTRIX* LEAF EXTRACT AND ASCORBIC

TABLE 6: IC50 VALUES OF THE DIFFERENT LEAF EXTRACTS OF CITRUS HYSTRIX IN NITRIC OXIDE SCAVENGING ASSAY

Standard/ extract	IC50 µg/ml
Methanol	92.2
Ethanol	84.9
Chloroform	93.7
Ascorbic Acid	11.2

Total Antioxidant Capacity: Total antioxidant capacity of the different extracts of *Citrus hystrix* was evaluated by the phosphomolybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid. Ethanol extract of *Citrus hystrix* was found to possess the highest total antioxidant capacity **Table 7**.

TABLE 7: TOTAL ANTIOXIDANT CAPACITY OF THE DIFFERENT EXTRACTS OF *CITRUS HYSTRIX*

Extract(s)	Total antioxidant Capacity (mg/gm), Ascorbic Acid Equivalent
Methanol	1.5±0.49
Ethanol	2.22±0.99
Chloroform	2.21±0.87

±S.D (standard deviation)

DISCUSSION:

Phytochemical Screening:

Preliminary phytochemical screening of the extracts of leaf of revealed the presence of alkaloid, carbohydrate, flavonoid, glycoside, phytosterols and tannin in each leaf extracts (methanol, ethanol & chloroform) of *Citrus hystrix* while saponins are absent **Table 1**.

In -Vitro* Thrombolytic Effect of *Citrus hystrix: Addition of 100µl Streptokinase (30,000I.U.), standard to the clots along with 90minutes of

incubation at 37°C, showed 37.43% clot lysis. Clots when treated with 100µl sterile distilled water (control) showed only negligible clot lysis (3.37%). The mean difference in percentage of clot lysis between standard & control was found to be statistically significant. In this study, methanol, ethanol, chloroform, extract of *Citrus hystrix*, showed 12.33%, 13.69%, and 12.19% of clot lysis respectively. In this study, the ethanol soluble fraction of ethanol extract of *Citrus hystrix* showed highest thrombolytic activity 13.69.14% (**Table 2**). However, *Citrus hystrix* leaf extract showed significant thrombolytic activity compared to standard.

Membrane stabilizing Activity:
In hypotonic solution the ethanol extract of *Citrus hystrix* inhibited 74.40% haemolysis of RBCs, compared to 93.24% inhibited by acetyl salicylic acid (0.10 mg/mL). The ethanol extract of *Citrus hystrix* significantly inhibited the haemolysis RBCs in hypotonic solution **Table 3**.

DPPH free radical scavenging assay: The free radical scavenging activity of different extracts of *Citrus hystrix* leaf was studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption. DPPH is a purple color dye having absorption maxima of 517nm and upon reaction with a hydrogen donor the purple color fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance methanol, ethanol and chloroform extracts showed maximum activity of 70.25%, 80.51% and 68.44% respectively at 800µg/ml, where as ascorbic acid at the same concentration exhibited 96.81 % inhibition **Fig. 1**.

Indicate the potency of scavenging activity. Standard ascorbic acid was found to have an IC_{50} of 15.08µg/ml. In comparison to standard ascorbic acid, methanol, ethanol, chloroform extract showed of 160.2, 182.25, 253.35µg/ml respectively.

Cupric Reducing Antioxidant Capacity (CUPRAC):
This method is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates increase in the antioxidant activity hence indicates the reducing power of the samples [22]. Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity [23]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid per-oxidation processes, so that they can act as primary and secondary antioxidants [24]. According to **Fig. 2** showed that in comparison to ascorbic acid methanol shows highest reducing capacity then chloroform and ethanol.

Total Phenolic Content: Phenol contents of the extracts were found to decrease in the following order: Methanol extract > Ethanol extract > Chloroform extracts **Table 4**. The results strongly suggest that phenols are important components of the tested plant extracts. Literature revealed that antioxidant activity of plant extract is mainly due to presence of phenol compounds, which may exerts antioxidant effects as free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators [25].

Total Flavonoid Content: Flavonoid contents of the extracts were found to decrease in the following order: Methanol extract > Chloroform extract > Ethanol extracts. Flavonoids play an important role in antioxidant system in plants. The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelating of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation [26]. Depending on their structure, flavonoids are able to scavenge practically all known ROS. The methanol extract of have been shown to possess highest flavonoids **Table 5**.

Nitric Oxide Scavenging Assay: The methanol, ethanol, chloroform extract showed maximum activity of 53.007%, 56.84% and 55.15% respectively at 100µg/ml, where as ascorbic acid at the exhibited 86.09% (**Fig. 3**). The IC_{50} values were found to be 92.3µg/ml, 84.9µg/ml, 93.7µg/ml and 11.2µg/ml for methanol, ethanol, chloroform and ascorbic acid respectively (**Table 6**).

Total Antioxidant Capacity: Total antioxidant capacity of the extracts was found to decrease in

the following order: Ethanol extract > Chloroform extract > Methanol extract (**Table 7**).

CONCLUSION: All the conducted experiments in the present study are based on crude extract and are considered to be preliminary and more sophisticate research is necessary to reach a concrete conclusion about the findings of the present study. The phytochemical screening revealed chemical constituents that form the foundation of their pharmacological activity. It can be concluded that the extracts of the above plants leaf can be used to design anti-thrombolytic agent due to its significant thrombolytic activity. Further work is needed to isolate the secondary metabolites and study thoroughly for more precise and accurate activities.

This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The use of these plants leaf in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases. Almost all extracts exhibited potential antioxidant activity. To sum up, these findings together demonstrate that *Citrus hystrix* leaf is an excellent candidate for further investigation of individual phenolic compounds, there *in vivo* antioxidant activity and the different antioxidant mechanisms and also appears to be a most promising candidate from which specific antioxidant bioactive products could be developed. Therefore, in depth extensive study should be an urgency to sort out bioactive compounds.

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