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Mild Extraction Technique for Flavonoids from Leaves of *Nypa fruiticans* and Chemical Analysis of Its Components

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Abstract: Eight flavonoid derivatives: rutin, quercetin–3-glucoside, quercetin, luteolin–7-glucoside, isorhmnetin–3-sulphate, kaempferol–3,7-diglucoside, luteolin and kaempferol have been extracted and characterized from Nipa palm. Mild extraction technique involving the use of HPLC-DAD-MS was used. The structures of the flavonoids were determined on the basis of mass spectroscopy. Separation of the crude extract by paper chromatography (PC) on forestall as solvent system gave one major yellowish brown spot which had R_f value of 3.9. The R_f value and maximum absorption from UV spectroscopy were the same as those of quercetin standard. The most prominent compound was quercetin followed by three others: kaempferol–3,7-diglucoside, luteolin–7-glucoside, and isorhmnetin–3-sulphate.

Key words: Nipa palm, flavonoids, extracts, spectroscopy, characterization.

1. Introduction

Nypa fruiticans, a mangrove palm, is one of the extra ordinary palms to be seen in the wild state of Australia, where palm is confined to a few small populations on the north-east and north coast [1]. It has also been reported to thrive in other countries especially in brackish waters and Nigeria is one of such [2-3]. Reports have shown that various products are gotten from this palm which include sugar from sap and nuts, feeds for livestock, craft items, alkaloids from the roots, etc. [4-6]. A preliminary work on the palm showed a UV max for flavonoids [7]. Most developing countries of the world especially Nigeria and other West African countries depend on plants for their traditional forms of medicine [8]. Medicinal plants as they are called are used in treating and preventing specific ailment and diseases and as such are considered to play a beneficial role in health care though based on proofs from western research [8]. It has been stated that hundreds of plant species are recognized as having medicinal values and the medicinal properties being present in one or all the parts of the plant: root, stem, bark, leaf, flower, fruit or seed [9]. It has been discovered that a striking characteristic of plants is that different chemical substances are obtained in members of even the same species in different areas [10]. The development and spread of resistance to the existing antibiotics by microorganisms call for increased efforts in the development of new antibiotics for treatment [9]. Many studies have shown that natural antioxidants from plants sources can effectively inhibit oxidation of food and reduce the risk of age dependent diseases [11]. Extraction of flavonoids from medicinal plants, fruits and vegetables have been studied extensively [12-14] because they present antioxidants with lower toxicity than synthetic antioxidants such as BHA and BHT [15]. New methods of separations have been developed for

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extraction of flavonoids [16-19]. In this study, a mild reported method [18] which utilizes a high resolution technique together with DAD chromophore and identification based on UV-visible absorption characterization will be used. This method preserves glycosidic linkages and their combinations with MS facilitate the identification of flavonoids and other glycosides.

2. Materials and Methods

2.1 Source of Samples

Nipa palm leaves were collected from the coastal region of Oron, Nipa Palm Utilization Project Centre, National Museum, Oron, Akwa Ibom State, Nigeria.

2.2 Separation by Paper Chromatography

1 g of the sample was extracted with 2 mL of HCl and the mixture was heated for 30 minutes at 100 ^{o}C. It was filtered and extracted with ethyl acetate, concentrated to dryness and taken up in 2 drops of ethanol. An aliquot was run on a whatman No. 1 filter paper using forestall. Forestall is the mixture of acetic acid-conc HCl-H_2O in the ratio 30:3:10.

2.3 Extraction

Extraction of flavonoids from Nipa palm leaves (estimated to weigh 0.2-0.8 mg) was done using methanol and water (1:1, v/v) for 2 hrs as reported [18]. The extract was centrifuged to separate particulate matter. The upper layer of solution was removed with a pipette for HPLC-DAD-MS analysis.

2.4 Analysis of Extract

Analysis of the palm extract was performed with an Agilent 1100 liquid chromatography system (HPLC) consisting of an automatic injector, a gradient pump, a HP series 110DAD and an Agilent series 1100 on-line atmospheric pressure elctrospray ionization MS. The flavonoid components were separated on a 2.1-mm-diameter Vydac C4 reversed phase column as previously described [18].

3. Results and Discussion

On separation using paper chromatography, a yellowish brown spot was noted on forestall with R_f value of 39. Comparing this 39 $R_f \times 100$ value with literature value, it was found to be probably quercetin [19].

The chromatographic profile of Nipa palm leaves is presented in Figs. 1 and 2. The elution profile in Fig. 1 shows eleven peaks observed out of which nine constituents were identified with two unknowns all under the class of flavonoids. The identification of the peaks was possible through the help of authentic samples, mass and UV spectral measurements as reported. The DAD profile of the visible eleven peaks of the elution profile is shown in Fig. 2. Their peaks eluting after 25 minutes are an evidence for classifying them under flavonoids or chalcones which do not absorb at 254 nm. From Table 1, six major peaks were identified as quercetin–4-glucoside (peak 2), quercetin (peak 3), luteolin–7-glucoside (peak 4), isorhmnetin–3-sulphate (peak 5), kaempferol–3,7-diglucoside (peak 6) and the sixth (peak 7) as an isomer of peak 1 and 6. Peak 1, a minor peak, was identified as rutin with absorbance of 7.2 and characteristic retention time of 25.538, UV max of 348 and molecular ion of 609 while peaks 10 and 11 as luteolin and kaempferol with corresponding absorbance of 2.6 and 2.5 respectively. From the six major peaks identified, peak 3 (quercetin) gave the highest absorbance of 53.2 followed by peaks 6 (kaempferol–3,7-diglucoside), 4 (luteolin–7-glucoside), 5 (isorhmnetin–3-sulphate), 7 (as an isomer of peak 1and 6) and 2 (quercetin–4-glucoside). All these flavonoids showed diagnostic MS spectral with intense molecular ions of daughter ions at 285 m/z for luteolin and 285 m/z for kaempferol.

Quercetin is a chemical pigment found mostly in the barks of a wide variety of plants which constitutes aglycone of the glycosides rutin and quercetin [20]. It has anti-inflammatory and antioxidant properties hence it has found a number of uses like antihistamine

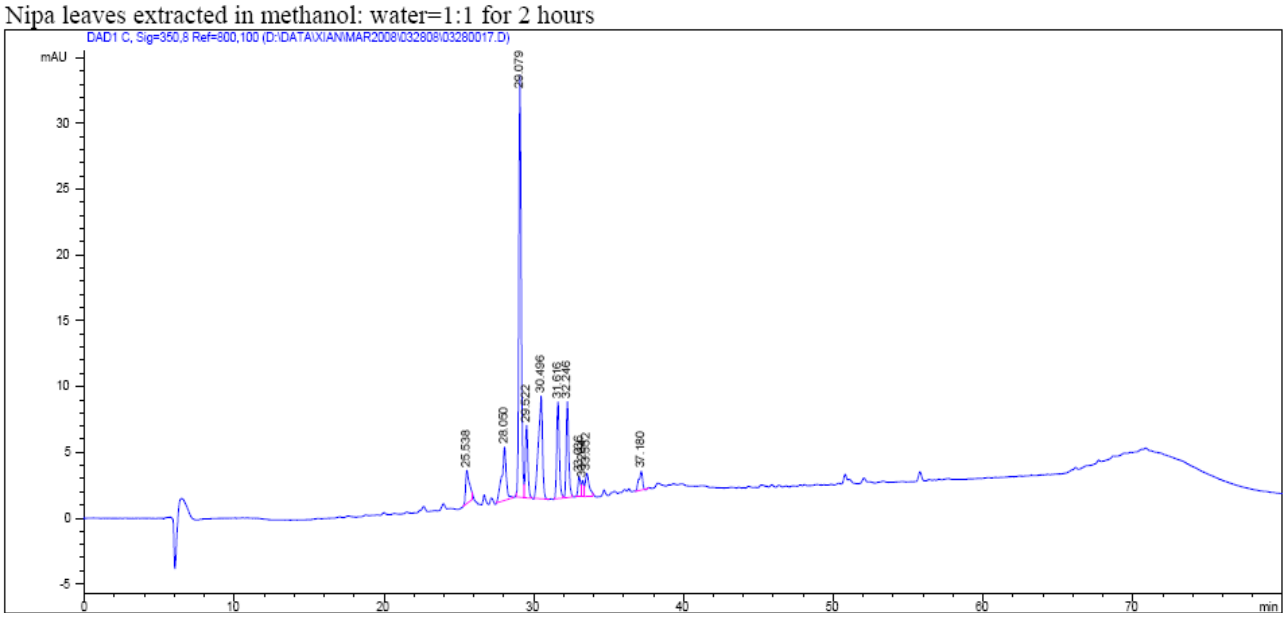


Fig. 1 Photographic profile of Nipa palm leaves.

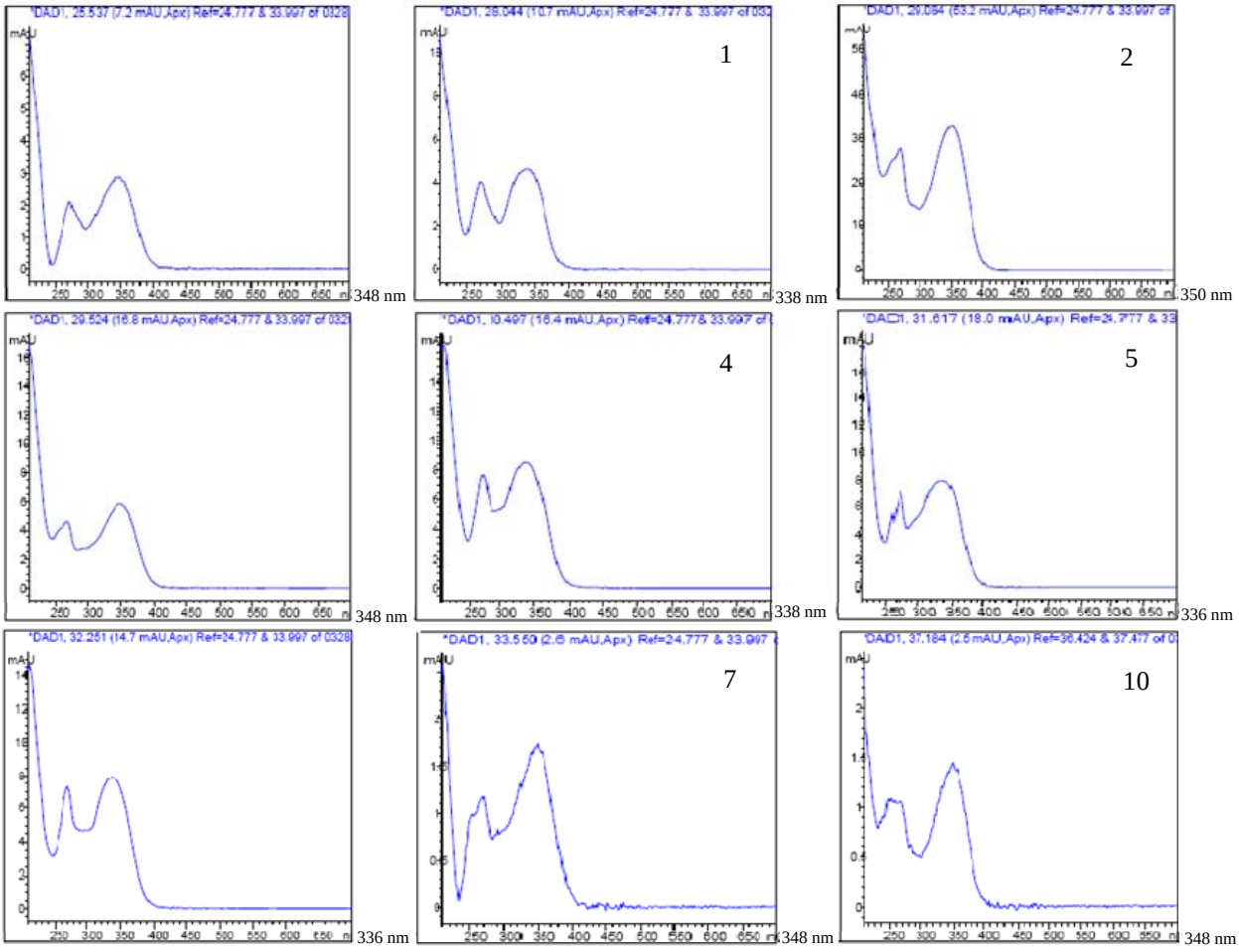


Fig. 2 DAD profile for the identified peaks in Nipa leaves.

Table 1 Identified compounds in leaf sample of Nipa palm by HPLC-DAD-MS.

Peak No.	Absorbance (mAU)	Retention time (RT) (min)	Max (nm)	$[M-H]^-$ (m/z)	Fragment ions (m/z)	Likely compound	References
1	7.2	25.538	348	609.1	463, 301, 153	rutin	[21-22]
2	10.7	28.050	338	463	302,153	quercetin-3-glucoside	[18]
3	53.2	29.079	350	301.0	153	quercetin	[18]
4	16.8	29.522	348	447	285	luteolin-7-glucoside	[17, 22]
5	16.4	30.496	338	396	315, 167	isorhmnetin-3-sulphate	[17, 22]
6	18.0	31.616	336	609.1	447, 285, 137	kaempferol-3,7-diglucoside	[21]
7	14.7	32.246	336	609.1	463, 301, 153	isomer of peaks 1 & 6	[21]
8	-	33.036	-	-	-	unknown	-
9	-	33.252	-	-	-	unknown	-
10	2.6	33.252	348	285	-	luteolin	[18, 22]
11	2.5	37.180	348	285	137	kaempferol	[21, 23]

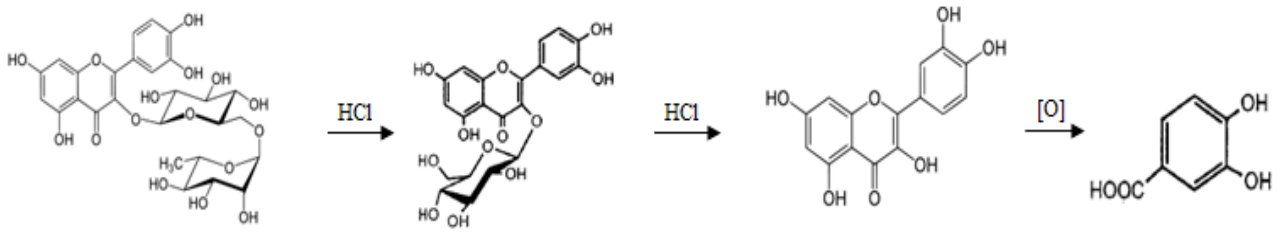
properties [24], helping control allergies and asthma [25], stabilizing small blood vessels [26], protecting against heart attack and strokes [27], helping prevent or treat different types of cancer [28], helping with symptoms of chronic prostatitis [29], relieving some of the neurologic complications of diabetes, etc. [30]. Luteolin was reported to be more effective in antioxidants properties than quercetin [31]. It is a naturally occurring flavonoid which exhibits a wide spectrum of pharmacological properties [32]. It was reported to have anti cancer properties by poisoning eukaryotic DNA topoisomerase I. [33], anti allergic properties and as a potent inhibitor of human mast cell activation through the inhibition of protein kinase C activation and Ca^{2+} influx [34]. Flavonoids generally have been reported to possess antioxidants properties; these inhibit cancer cell proliferation in vitro [35], anti viral, anti parasitic, anti cancer activities. A chalcone type extract of UV max 348 nm and molecular mass of 448 and peak 4 has been identified as luteolin–7-glucoside. This compound is one of the most common yellow dyes and occurs in many species of plant dyestuff [20]. Acid hydrolysis also yields aglycone, luteolin.

Flavonols generally refer to 3-hydroxyflavones of which the most common members are derivatives of quercetin [36]. Quercetin 3-sulfate and isorhamnetin 3-sulfate (M of 382 and 396, respectively) are the most distinctive feature of the flavonol-type dyes [37]. It was reported that the same species of substituted 3-hydroxylflavone which were identified an archaeological samples were same as gotten from *F. haumanii* specimen [38]. In a pioneering work on natural dyestuff, it was reported that flavonoids dyes have been utilized in textile [39].

4. Possible Mechanism of Fragmentation

The possible mechanism fragmentation is shown in Fig. 3. It was evident that all the aglycones are 3-hydroxy flavones and all seem to have glycosyl or sulphate groups on the 3-hydroxyl group as shown by their UV max which is typical of 3-o-substituted flavones except for peak 4 and 10. By a way of contrast, glycosides of luteolin lack a 3-hydroxyl group and its UV max did not shift when the glycoside was hydrolysed.

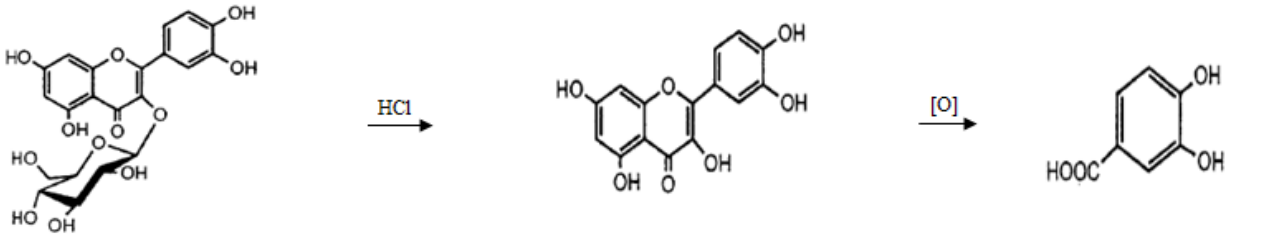
Peak 1



Rutin 610 | Quercetin-3-glucoside 464 | Quercetin 302 | 3, 4-Dihydroxybenzoic acid 154

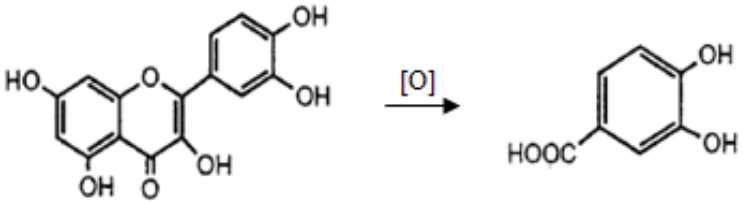
(Fig. 3 continued)

Peak 2



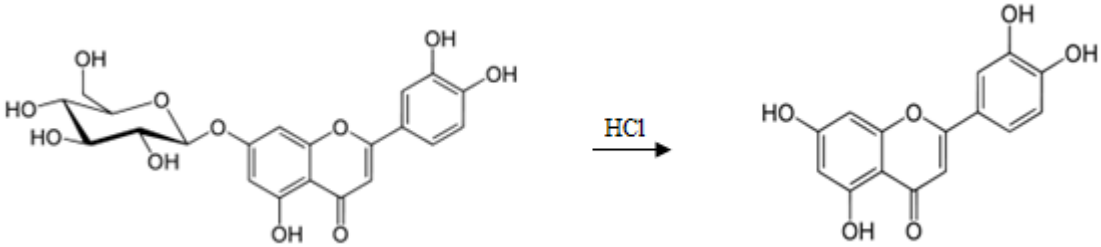
Quercetin-3-glucoside 464 | Quercetin 302 | 3,4-Dihydrobenzoic acid 154

Peak 3



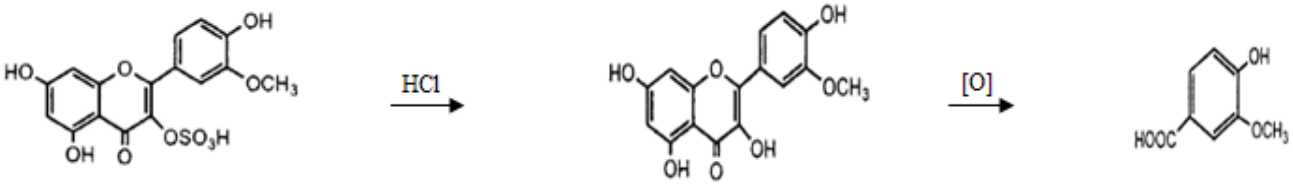
Quercetin 302 | 3, 4-Dihydrobenzoic acid 154

Peak 4



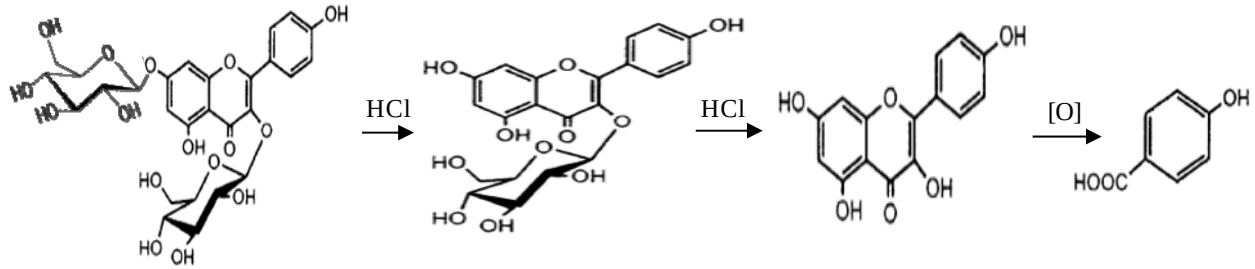
Luteolin-7-glucoside 448 | Luteolin 286

Peak 5



Isorhamnetin-3-sulfate 396 | Isorhamnetin 316 | 4-Hydroxy-3-methoxybenzoic acid (Vanillic acid) 168

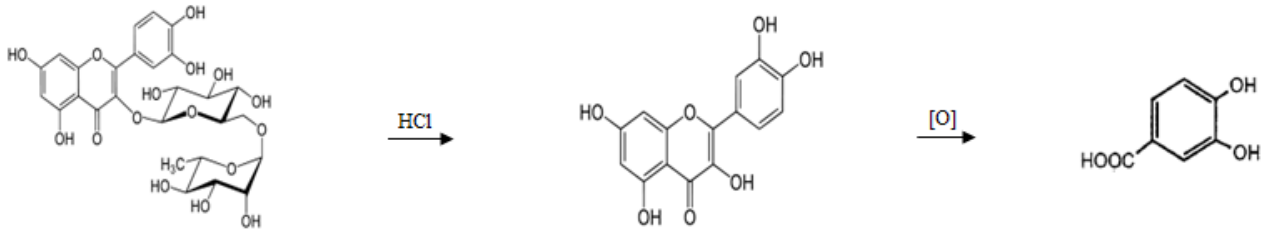
Peak 6



Kaempferol-3,7-diglucoside 610 | Kaempferol-3-glucoside 448 | Kaempferol 286 | 4-Hydroxybenzoic acid 138

(Fig. 3 continued)

Peak 7

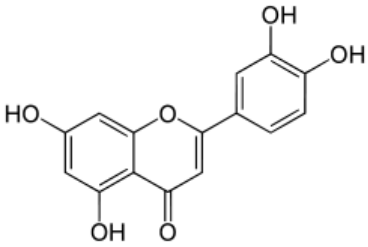


Isomer of rutin
610

Quercetin
302

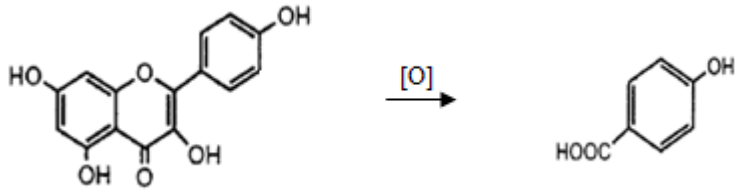
3, 4-Dihydroxybenzoic acid
154

Peak 10



Luteolin
286

Peak 11



Kaempferol
286

4-Hydroxybenzoic acid
138

Fig. 3 Schematic representations of fragmentation of the identified peaks 1-7, 10-11.

5. Conclusion

The phytochemical results showed that this palm is a good medicinal plant rich in flavonoids. The flavonoids content for instance could be used as antioxidants, anti histamine, and anti-inflammatory properties and are effective against a wide variety of diseases [32]. For instance it has been reported that since ancient times man has utilized alkaloids as medicine. The results have been showed that Nipa palm leaves contain high percentage of flavonoids. The result of the paper chromatography (PC) separation on forestall as solvent system gave one major yellowish brown spot which had R_f value of 3.9. The R_f value and maximum absorption from UV spectroscopy were the same as those of quercetin standard. Also from the result of the HPLC-DAD-MS analysis, the most prominent compound was quercetin followed by kaempferol–3,7-diglucoside, luteolin–7-glucoside, and isorhmnetin–3-sulphate. Quercetin in particular has been noted for a number of uses like antihistamine, helping control allergies and asthma, stabilizing small blood vessels, protecting against heart attack and strokes, helping prevent and treat different types of cancer, helping with the symptoms of chronic prostatitis (swelling of the prostrate gland), and relieving some of the neurologic complications of diabetes.

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