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1 **Antioxidant and genoprotective activity of selected *cucurbitaceae***
2 **seed extracts and LC-ESIMS/MS identification of phenolic**
3 **components**

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20 **ABSTRACT**

21 Cucurbitaceae are one of most widely used plant species for human food but lesser
22 known members have not been examined for bioactive components. The purpose of
23 this study was to evaluate the antioxidant and genoprotective activities from three
24 cucurbitaceae seeds extracts and to identify phenolic components by LC-ESIMS/MS
25 analysis. From the results, the yield of seeds extract was 20-41% (w/w) and samples
26 had 16-40% total phenols as gallic acid equivalents (GAE). Compared with methanol
27 solvent, using acidified methanol led to increased extraction yield by 1.4 to 10-fold,
28 higher phenolic content (149.5±1.2 to 396.4±1.9 mg GAE/g), higher DPPH radical
29 quenching and enhanced genoprotective activity using the pBR322 plasmid assay. LC-
30 ESI-MS/MS analysis led to identification of 14-17 components, based on authentic
31 standards and comparison with literature reports, as mainly phenolic acids and esters,
32 flavonol glycosides. This may be the first mass spectrometric profiling of polyphenol
33 components from cucurbitaceae seeds. **(140words)**

34 **Keywords:** *Cucurbitaceae* seeds, total phenols, phenolic glycosides, genoprotective;
35 antioxidant; pBR322 plasmid; LC-ESI-MS/MS analysis.

36 **Highlights:**

37 Cucurbitaceae seeds are novel sources of flavonol glycosides

38 Cucurbitaceae seeds are sources of phenolic acid and esters

39 Acidification improves aqueous methanol recovery of cucurbitaceae glycosides

40 Extracts from Cucurbitaceae seeds possess antioxidant and genoprotective activity

41 **Chemical compounds studied in this article**

42 4-O-Feruloylquinic acid (PubChem CID: 6171347)

43 Caftaric acid (PubChem CID: 6440397)

44 Chicoric acid (PubChem CID: 5281764)

45 Isoquercetin, quercetin 3-O-glucoside (PubChem CID: 5280804)

46 Kaemferol (PubChem CID: 5280863)

47 Myricetin (PubChem CID: 5281672)

48 Quercitrin (Quercetin 3-O-rhamnoside), PubChem CID: 535943

49 Rosmarinic acid (PubChem CID: 5281792)

50 Synapic acid (PubChem CID: 10743)

51 Syringic acid (PubChem CID: 10742)

52

53 # 1. Introduction

54 *The Cucurbitaceae* family contains one of most the widely used plant species for human
55 food. The family comprises of 120 genera and 825 species of which 17 genera and 32
56 species are found in Pakistan. Leaves, fruits and dried seeds from *Cucurbitaceae*
57 (pumpkin, cucumber, melon, watermelon, squash and gourds) are widely consumed.
58 Plants belonging to the *Cucurbitaceae* family may possess pharmacological properties
59 (Talukdar & Hossain, 2014; Vijayakumar, Eswaran, Ojha, Rao Ch & Rawat, 2011),
60 including antidiabetic role (Chandrasekar, Mukherjee & Mukherjee, 1989; Huseini,
61 Darvishzadeh, Heshmat, Jafariazar, Raza & Larijani, 2009; Rashidi, Mirhashemi,
62 Taghizadeh & Sarkhail, 2013), anti-ulcer role, analgesic, nephro-protection (Jain &
63 Singhai, 2010), and anticancer effects (Vijayakumar et al., 2011).Pumpkin (*Cucurbita*
64 *pepo* L) was extensively investigated for uses in herbal therapy (Abdel-Rahman, 2006)
65 and as a source of bioactive food compounds (Veronezi & Jorge, 2012). Less well-
66 investigated cucurbitaceae are now receiving attention related to both food and
67 medicinal applications (Milind & Kulwant, 2011; Talukdar & Hossain, 2014).

68 Phytosterols were identified in the *Cucurbitaceae* as biologically active
69 components with antiviral activity (Akihisa, Ghosh, Thakur, Rosenstein & Matsumoto,
70 1986; Akihisa, Inada, Ghosh,Thakur, Rosenstein, Tamura et al, 1988; Akihisa, Kimura,
71 Kasahara, Kumaki, Thakur & Tamura, 1997; Akihisa, Ogihara, Kato, Yasukawa, Ukiya,
72 Yamanouchi et al, 2001). Antioxidant capacity of Cucurbitaceae seeds was correlated
73 with total phenols (Achu, Fokou, Kansci & Fotso, 2013; Ismail, Chan, Mariod & Ismail,
74 2010; Talukdar & Hossain, 2014). Koike, Li, Liu, Hata, and Nikaido (2005) and also

75 Li, Xu, Dou, Chi, Kang and Kuang (2009) identified 5-8 novel phenolic glycoside
76 derivatives of 4-hyroxy benzyl alcohol from different varieties of *Cucurbitaceae* seeds.

77 There is growing interest in the characterization of plant polyphenols (de Rijke,
78 Out, Niessen, Ariese, Gooijer & Brinkman, 2006; Naczk & Shahidi, 2004; Naczk &
79 Shahidi, 2006; Pérez-Jiménez, Neveu, Vos & Scalbert, 2010). However, phenolic
80 compounds from Cucurbitaceae seeds have not been thoroughly investigated. We
81 previously applied solid phase extraction (SPE) for the isolation of flavonols from plants
82 of medicinal and food value (Sultana & Anwar, 2008; Sultana, Anwar & Przybylski,
83 2007). The purposes of the study reported in this paper were to, evaluate total phenols
84 content, free radical quenching activity, and genoprotective activities from cucurbitaceae
85 seed extracts. The polyphenol enriched extracts were subjected SPE and LC-
86 ESIMS/MS analysis. This is the first application of LC-MS analysis of cucurbitaceae
87 seed phenols.

88 2. Materials and methods

89 2.1. Chemicals and Reagents

90 Samples of 2,2-diphenyl-1-picryl-hydrazl (DPPH) were obtained from Aldrich Chemical
91 Co. (Steinheim, Germany). Folin–Ciocalteau reagent and gallic acid were purchased
92 from Sigma Chemical Co. (St. Louis, USA). Butylated hydroxytoluene (BHT) and
93 ascorbic acid were procured from Merck (Darmstadt, Germany) while dimethylsulfoxide
94 (DMSO) was from AppliChem (Darmstadt, Germany). pBR322 DNA plasmid was
95 purchased from Fermentas. All chemicals were of HPLC or LC/MS grades.

2.2. Extraction of cucurbitaceae seed phenols

Fruits of Momordica *dioica (*Spinney gourd, Jungli karela*), Citrullus colocynthus* L. (bitter cucumber, desert gourd, egusi,) and *Cucumis melo var. agrestis (mouse melon, chibber, ucado melon,*) were collected from local farms in Faisalabad, Pakistan. The subject species were selected based on their multiple medicinal benefits and their availability in Pakistan. The plants were identified from the Department of Botany, University of Agriculture, Faisalabad, Pakistan. Current nomenclature is available from the USDA germ plasm database (USDA-ARS National Genetic Resources Program, 2015). Seeds were manually separated, washed, shade dried and ground to fine powders. The ground seeds were extracted in an orbital shaker (PA 250/25-H) by sample to solvent ratio of 1:10 (w/v) with methanol/water (70%, 50% and 30% v/v) at room temperature for 24 hours. Seed powders were not defatted prior to antioxidant extraction to avoid losses of nonpolar constituents. Acidified methanol extraction was performed as described previously (Abdel-Aal el & Hucl, 2003; Kim, Kim, Koh, Kim, Lee & Kim, 2008; Sultana et al., 2008; Takeoka, Dao, Full, Wong, Harden, Edwards et al, 1997) with modification. Briefly, samples (10g) of powdered seed were shaken with 100 mL acidified methanol (methanol + 0.5M, 1.0M and 2.0M HCl; 70: 30% v/v) at room temperature for 24 hours. The acidified methanol and non-acidified methanol extracts were concentrated to complete dryness under reduced pressure and stored at 4°C until further analysis.

116 **2.3. Total Phenolic Contents**

117 Non-defatted seed powders were used in this study. Total phenolic contents of all
118 extracts were analyzed using Folin–Ciocalteu reagent. Methanol/water seed extracts
119 (0.5 mL having 1mg dry extract) was mixed with sodium carbonate (2 mL, 7.5%) and
120 Folin–Ciocalteu reagent (2.5 mL, 10%). The mixture was incubated for 30min at room
121 temperature, then absorbance was recorded at 765nm using a UV-VIS
122 spectrophotometer (IRMECO, Geesthacht/Germany, Model 5000). Total phenolic
123 contents were quantified based from absorbance measurements (Abs) and standard
124 curve for gallic acid (2-200 ppm) using the relations below;

125

126 Total phenols (mg-GAE/ g sample) = $\frac{Abs}{m} * V_{ex} * D_F * \frac{1}{W}$

127 where, m (l/mg) = slope from the calibration graph, DF = Dilution factor for sample
128 before assay (1 if undiluted), Vex = Original volume of sample extract and W = dry
129 weight of seed extract (g).

130

131 **2.4. DPPH Free Radical Scavenging Activity**

132 The antiradical activity of each extract was evaluated following a spectrophotometric
133 DPPH method (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos & Byrne, 2006).
134 Methanolic solutions of each extract (3 mL) at varying concentrations (1-5000µg/mL)
135 were added to methanolic solution of DPPH (1 mL, 0.1mM) and allowed to stand in the
136 dark for 30min at room temperature. Then the absorbance of solution was measured at

137 517nm (IRMECO 5000) and antioxidant activity was calculated as percentage inhibition

138 of DPPH free radical using the following equation below;

139 % reduction (DPPH) = $\left[1 - \frac{Absorbance\ sample}{Absorbance\ of\ DPPH\ soln}\right] * 100$

140 the concentration of compound to produce 50% inhibition of DPPH (IC_{50}) for DPPH free

141 radical scavenging were calculated using linear regression analysis; IC5= 50/m.

142 **2.5. PBR322 DNA plasmid protection assay**

143 Genoprotective effects of plant extracts were evaluated qualitatively on supercoiled

144 pBR322 DNA plasmid following a previous method (Tepe, Degerli, Arslan, Malatyali &

145 Sarikurkcu, 2011). In this assay, the protection ability of plant extracts against damage

146 caused by H_2O_2 and UV radiations on DNA plasmid was measured using agarose gel

147 electrophoresis. The irradiation experiments were conducted in Eppendorf tubes

148 containing pBR322 DNA plasmid (3 μL, 172 ng/μL), plant extract (5 μL, in varying

149 concentrations of 5, 10, and 20 mg/mL) and H_2O_2 (2 μL, 30%). A negative control

150 without plant extracts was also run along with sample reactions. All Eppendorf tubes

151 were exposed to UV radiations for 15 min to breakdown the supercoiled DNA plasmid.

152 For electrophoresis analysis, the reaction mixtures were transferred to 0.8% agarose

153 gel along with loading dye (6x) dissolved in Tris Acetate–EDTA buffer (1x). Gels were

154 photographed using gel documentation system (GeneGenius, SYNGENE) after staining

155 with ethidium bromide (0.5μg/mL).

156 **2.6. Purification and LC/MS of optimized seed extracts**

157 The phenolic compounds present in seed extracts optimized as above (Section 2.2-2.5)
158 were investigated by LC/MS technique. First seed extracts were purified and
159 concentrated by solid phase extraction (SPE) and then subjected to LC/MS analysis.

160 *2.6.1. Solid Phase Extraction (SPE)*

161 A multichannel SPE cartridge (ThermoScientific) with Strata C-18 columns and vacuum
162 pump was applied to eliminate non-phenolic compounds. The pre-conditioning of SPE
163 columns was done by 1 mL methanol followed by 1 mL distilled water, in order to
164 remove trapped air and to activate ligands present on sorbent surface. Then, 3.5 mL of
165 plant extracts, diluted in methanol/water (50/50), were loaded onto SPE columns.
166 Washing was performed with 1 mL of distilled water and 1 mL methanol/water (30/70) in
167 order to elute all the impurities without affecting sample analytes. A full vacuum drying
168 was applied for about 5-10 min to remove residual solvent. To recover phenols,
169 cartridges were eluted 1 mL acetonitrile, then 2 mL methanol and finally 2 mL of 5%
170 formic acid in methanol. All three fractions were combined before subjecting to LC/MS
171 (Sun, Liang, Bin, Li & Duan, 2007)

172 *2.6.2. LC-ESI-MS/MS Analysis*

173 Plant extracts purified by SPE were subjected to LC-ESI-MS/MS analysis. This analysis
174 was carried out on liquid chromatography coupled with mass spectrometry (LC/MS)
175 using a ThermoFisher system in which HPLC (Surveyor) system was equipped with
176 linear ESI-Ion Trap (LTQ XL) Mass Spectrometer (ThermoFisher Scientific, San Jose,

177 CA, USA). Usually, 5µL of sample was injected via an autosampler (Surveyor
178 autosampler plus) in to the HPLC system (Surveyor) equipped with reverse phase C-18
179 column (Phenomenex 250mm, 5 µm particle size). Sample elution was carried out at
180 flow rate of 5 ml/min using gradient elution comprising Solvent A (water: acetonitrile:
181 trifluoroacetic acid ratio 90:10:0.1% (v/v)) and solvent B (water: acetonitrile:
182 trifluoroacetic acid ratio 10:90:0.06% (v/v). Elution was performed using the following
183 gradient: 0-10 min: 10-35% B, 10-20 min: 35-42 % B and 20-30min: 42-100% B. A
184 photodiode array was used as detector. Prominent peaks were analyzed by mass
185 spectrometer (LTQ XL ThermoFisher Scientific) using atmospheric pressure
186 electrospray ionization (ESI) probe at negative ion mode. Identification of phenols was
187 conducted under full scan mode in the range of 100-600 m/z. MS^2 analysis for each
188 parent ion peak was performed at different Collision Induced Dissociation (CID) powers.
189 X-calibur 1.4 software was applied for calibration of MS data (Sun et al., 2007).

190 **2.7. Statistical analysis**

191 Data are presented as mean ± S.D. of three parallel determinations. Significant
192 difference were analyzed by one way analysis of variance (ANOVA) followed by
193 Duncan's Multiple Range test using MSTAT-C software (version 1.3). Differences
194 among values for were considered statistically significant with $P < 0.05$.

195 ## 3. Results and discussion

196 ### 3.1. Extraction yield by mass

197 Acidified methanol produced a higher yield of seed extract compared to aqueous
198 methanol (Table 1). The mass-yield of extract ranged from 4.0% using aqueous
199 methanol to 72.6% for acidified methanol as solvent. Among three *cucurbitaceae*
200 species, *Momordica dioica* exhibited highest extract yield (28.8±0.2 %) for methanol
201 extraction while *Cucumis melo var. agrestis* showed the highest extract yield
202 (72.6±1.0%) in case of acidified methanol extraction. The extraction yields for various
203 solvents follow the order: 70% methanol > 50% methanol > 30% methanol for non-
204 acidified methanol extraction; and 2.0 M acidified methanol > 1.0 M acidified methanol >
205 0.5 M acidified methanol for acidified methanol extraction. This different extraction
206 efficiency could be explained by large differences in solubility of various phytochemicals
207 present in these plant species including oils, carbohydrates as well as polyphenols.
208 Many phytochemicals are more soluble in methanol rather than in aqueous medium.

209 ### 3.2. Total Phenolic Contents

210 Phenolic acids and polyphenols are important plant secondary metabolites responsible
211 for plant antioxidant activity. Plant phenols can produce antioxidant capacity by a variety
212 of mechanisms, including free radical scavenging, single electron reductions and metal
213 ion chelation (Huang, Ou & Prior, 2005). Previous reports showed that the total phenols
214 content of whole Cucurbita seeds was 0.34-0.4% (w/w) expressed as gallic acid
215 equivalents (Achu et al., 2013; Ismail et al., 2010). In this study (Table 1) the total

216 phenolic contents for *Cucurbitaceae seeds extracts* ranged from 16% to 40% (w/w)
217 expressed as gallic acid equivalents. For the range of solvents employed in this study,
218 increasing the methanol concentration from 50 to 70 % v/w and acidification of methanol
219 increased the extraction phenolic components. Acidified methanol has been
220 demonstrated to improve the extraction of plant flavonoid glycosides and aglycones,
221 partly due to increasing hydrophobicity of solvent and low pH suppression of polyphenol
222 oxidases (Acosta-Estrada, Gutierrez-Uribe & Serna-Saldivar, 2014; Haghi & Hatami,
223 2010; Kim et al., 2008; Koh, Youn & Kim, 2014). The mild acidified methanol extraction
224 applied in this investigation is not believed to produce a loss of glycosides (see Section
225 3.5). Moreover, many polyphenols are ionizable (pKa~7-9) and a low pH solvent would
226 increase the concentration of uncharged species and improve extractability (Wong,
227 Cheung, Lau, Bolanos de la Torre & Owusu-Apenten, 2015).

228 **3.3. DPPH free Radical Scavenging Activity**

229 DPPH is a stable free radical with deep violet color. Radical quenching agents react
230 with DPPH whereby this is reduced to a non-radical yellow colored molecule. In Table 1,
231 acidified methanol plant extracts exhibited significantly ($P \leq 0.05$) higher free radical
232 scavenging activity than non-acidified methanol samples which is consistent with the
233 higher total phenols content (Section 3.2). Previous investigations demonstrated a
234 correlation between total phenols content and DPPH radical quenching activity (Sultana
235 et al., 2007).

236

237 **3.4. pBR322 DNA plasmid DNA protection assay for oxidative stress**

238 Protective effects of 0.5N acidified methanol and 70% aqueous methanol extracts of
239 *Momordica dioica, Citrullus colocynthus* and *C. melo varagrestis* were evaluated by UV
240 and •OH induced breaks in pBR322 DNA plasmid as *in vitro* assay (Gandhi & Nair,
241 2005; Sevgi, Tepe & Sarikurkcu, 2015). H_2O_2 in the presence of UV radiations generate
242 hydroxyl radicals that initiate a chain reaction leading to the breakdown of sugar-
243 phosphate backbone of DNA. Hydroxyl radicals also react with nitrogenous bases of
244 nucleic acid, thus, breaking the supercoiled form into linear and open circular form.
245 Figures 1(a), (b) and (c) represent electropherograms for DNA nicking assay of
246 *Momordica dioica, Citrullus colocynthus* and *C. melo varagrestis,* respectively; the
247 direction of migration is from lower to upper part of the electropherograms. The faster
248 moving band in lane A corresponds to supercoiled circular DNA and slower moving
249 band represents open circular DNA following treatment with H_2O_2 and UV rays. Lanes
250 C-E represent DNA co-treatment with 0.5M acidified methanol extracts of *Momordica*
251 *dioica* (Fig. 1a)*, Citrullus colocynthus* (Fig. 1b) and *C. melo varagrestis* (Fig. 1c) at
252 concentrations of 5, 10 and 20 mg/mL, respectively. Similarly, lanes F-H show DNA co-
253 treatment by 70% aqueous methanol extracts at concentrations of 5, 10 and 20 mg/mL,
254 respectively. Overall electrophoretic patterns are consistent with the protection
255 supercoiled DNA form in the presence of 0.5 M acidified extract but the DNA protection
256 was less clear following treatment with 70% methanolic extract (lanes F-H).
257

258 **3.5. LC-ESI-MS/MS Analysis**

259 Tables 2-4 summarize the LC-ESI-MS/MS characterization of 0.5 M acidified methanol
260 extracts. Peaks were identified by reference to retention times, fragmentation patterns
261 and by comparison with published libraries; peaks were also authenticated from
262 molecular weight estimates and supplementary data from the Phenol-Explorer database
263 (Neveu, Pérez-Jiménez, Vos, Crespy, du Chaffaut, Mennen et al., 2010; Pérez-Jiménez
264 et al., 2010).

265 HPLC analysis of SPE isolates from *Momordica dioca* led to identification of fourteen
266 components with a mass range of 160-600 *amu* (Table 2). Figure 2 shows a sample
267 LC/MS data for *Momordica dioca* extracts; Peak 13 (RT 26.46 min) showing MS peak at
268 m/z 311.07 (Figure 2) indicated the presence of caftaric acid. The parent ion peak was
269 subjected to CID fragmentation to give three daughter ions at m/z 179, 149 and 135. First
270 peak (m/z 179) corresponded to molecular ion of caffeic acid by losing tartaric acid
271 residue, second peak (m/z 149) remained unidentified while a third peak (m/z 135)
272 corresponded to a decarboxylated caffeic acid.

273 The components isolated from Cucurbita seed extracts could be grouped into two
274 broad classes, (a) phenolic acids and esters and (b) flavonoids and flavonoid
275 glycosides. The phenolic acids mainly hydroxycinnamic acid derivatives were (Table 2);
276 methyl ellagic acid (peak 4), ellagic acid (peak 7), rosmarinic acid (peak 10), caftaric acid
277 (peak 13) and 4-feruloylquinic acid (peak 15). The flavonols were represented by
278 galangin (peak 6), quercetin (peak 8) and myricetin (peak 14). Flavonol glycosides were
279 identified as naringenin 7-O-glucoside (peak 3), apigenin 7-O-glucuronide (peak 11),

280 myricetin 3-O-glucoside (peak 12), quercitrin (peak 17) and myricitrin or myricetin-3-O-
281 rhamnoside (peak 18). As an example, naringenin-7-O-glucoside (peak 3) with a RT of
282 4.23 min produced an MS peak at m/z 433.11. This parent ion then fragmented to give
283 two peaks at m/z 271 *amu* (by loss of glucose residue) and other at m/z 153 which is
284 characteristic of naringenin as reported by previously (Pfundstein, El Desouky, Hull,
285 Haubner, Erben & Owen, 2010). The other glycosides were identified similarly
286 according their retention time and MS/MS patterns. Quercetrin (quercetin 3-O-
287 rhamnoside) was the highest concentration (532 ppm) amongst the identified phenolic
288 constituents of *Momordica dioca.* Caftaric acid (caffeic acid ester with tartaric acid;
289 460.16 ppm); myricetin aglycone (439.78 ppm); myricitrin (myricetin 3-O-rhamnoside;
290 423.19 ppm) and 4-feruloylquinic acid (423.48 ppm) were also present in considerable
291 higher concentrations.

292 LC-MS/MS analysis results for C*itrullus colocynthus* (Table 3) showed that
293 components were, (a) phenolic acids or phenolic acid conjugates or (b) flavonol
294 glycosides. The phenolic acids were represented by vanillic acid (peak 1), sinapic acid
295 (peak 4), ferulic acid (peak 5) and ellagic acid (peak 6). Examples of phenolic acid
296 conjugates (peaks 3, 9, and 19) were protocatechuic (peak 3), caffeoyl glucose (peak 9)
297 and chicoric acid (peak 19). Flavonol (peaks 8, 12 and 15) and flavonol glycosides
298 apigenin-7-glucoside (peak 10), myricetin 3-O-glucoside (peak 11), kaempferol-3-
299 rutinoside (peak 13), myrictrin (myricetin 3-O-rhamnoside; peak 14) and isoquercitrin
300 (quercetin 3-O-glucoside; peak 16) were also present. Quantitative analysis showed
301 (Table 3) that dicaffeoyl tartaric acid (chicoric acid; 454.92 ppm), sinapic acid (409.51

302 ppm) kaempferol (394.08 ppm), isoquercetrin (392.62 ppm) and luteolin (329.74 ppm)
303 were present in quite high concentrations.

304 For *Cucumis melo var. agrestis* LC/MS analysis of material eluted from SPE
305 separation indicated twelve phenolic compounds comprising, (a) phenolic acids and
306 their conjugates (peaks 2, 6, 3, 4, 8, 10 and 14) and (b) flavonol and their derivatives
307 (peaks 7,9, 13). The former group were represented by syringic acid (peak 2), ferulic
308 acid (peak 6), methyl gallate, (peak 3), sinapic acid hexoside (peak 4), caffeoyl glucose
309 (peak 8), 1,6-digalloyl glucose (peak 10), glycosyringic acid (peak 14). The flavonoids
310 and their derivatives included chrysin (peak 7), bis-methylated quercetin (peak 1),
311 quercitrin (peak 9), isoquercitrin (peak 11), malvidin-3-O-glucoside (peak 13). From the
312 quantitative analysis (Table 4) then glycosyringic acid (445.60 ppm), malvidine-3-O-
313 glucoside (399.61 ppm), quercetrin (344.29 ppm) and bis-methylated quercetin (343.71
314 ppm) were most abundant compounds of all bioactive compounds found acidified
315 methanol extract of *Cucumis melo varagrestis.* Other phenolic compounds were also
316 present in quite reasonable concentrations (250-300ppm) as demonstrated in Table 4.

317 Acidified methanol solvent extraction is thought to avoid the enzyme catalyzed
318 oxidation of polyphenols as well as increasing the release of compounds bound by
319 physical forces (Abdel-Aal el et al., 2003; Acosta-Estrada et al., 2014; Kim et al., 2008;
320 Sultana et al., 2008; Sultana et al., 2007). Indeed, the use of mild acidified methanol
321 extraction is a common approach for recovery of anthocyanin glycosides. By contrast,
322 conversion anthocyanin to the aglycone state form required treatment with 6N HCl at
323 100 ^{o}C for 30 min – 4 hours (Abdel-Aal el et al., 2003; Kim et al., 2008; Takeoka et al.,
324 1997). Interestingly, whilst *Momordica dioica* contained quercitrin (quercetrin-3-O-

325 rhamnoside) it was quercetrin-3-O-glucoside (isoquercitrin) derivative which was found
326 in *Citrullus colocynthus*.

327 Some study limitations with relevance for data interpretation are worth noting.
328 Though polyphenolic compounds from Cucurbitaceae seeds were profiled, it is not
329 certain that extracts purified by SPE are representative of all classes of compounds.
330 Tables 2-4 show the predominant components isolated under the present method
331 (acidified methanol extraction and SPE) were flavonol/ flavonol glycosides and phenolic
332 acids and derivatives. Some flavanones (hesperetin) and flavones (chrysin) occurred in
333 lower concentrations. Other classes of polyphenols were not presented in the SPE
334 isolated sample. A further interesting feature is that though flavonol glycosides were
335 present in higher concentrations compared to aglycones, the former did not reach 99-
336 100% glycoside distribution noted in other sources (Pérez-Jiménez et al., 2010). The
337 presence of quercetin glycosides in high quantities is notable as these have enhanced
338 bioavailability compared to the corresponding aglycones (Crespy, Morand, Besson,
339 Manach, Démigné & Rémésy, 2001). The current investigations also do not allow
340 estimates of the different components in unextracted foods of a fresh weight basis.

341 4. Conclusions

342 The antioxidant and genoprotective activity for polyphenol extracts of *Momordica*
343 *dioica, Citrullus colocynthus* and *C. melo var. agrestis* were analyzed. The acidified
344 methanol extraction yielded greater amounts of extracts, a higher polyphenol contents,
345 higher antioxidant activity and increased genoprotective activity. *Citrullus colocynthus*

346 showed the highest phenolic contents and thus highest antioxidant potential. To our
347 knowledge this is the first investigation of Cucurbit seed polyphenol constituents using
348 LC-MS/MS analysis. The results indicated the presence of phenolic acids, flavonoids
349 and flavonoid glycosides. It is concluded that Cucurbita seeds may be important
350 sources of antioxidant compounds and also a range of phytochemicals with possible
351 nutraceutical uses.

352

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357 **Conflict of interest**

358 All authors declare no conflict of interest.

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482 # List of Tables

483 ## List of tables (4)

484

485 **Table 1**: Extract yields (%age), total phenolic contents (GAE, mg/g) and %age DPPH
486 scavenging activity of non-hydrolyzed and hydrolyzed extracts of *M. dioica, C.*
487 *colocynthus* L. and *C. melo varagrestis*.

	Momordica dioica			***Citrullus colocynthus* L.**			***Cucumis melo varagrestis***		
solvent	Extract Yield (%)[A]	TPC[B]	%age Scavenging[C]	Extract Yield (%)[A]	TPC[B]	%age Scavenging[C]	Extract Yield (%)[A]	TPC[B]	%age Scavenging[C]
70% Meth	28.8±0.2 [b]	228.6 ±1.9 [d]	91.62±1.3 [b]	15.8±0.2 [d]	251.4 ±2.3 [b]	89.21 ±1.1 [d]	5.4 ±0.07 [d]	41.6 ±0.9 [d]	72.76±1.4 [d]
50% Meth	21.5±0.3 [c]	220.2 ±1.7 [e]	89.56±1.4 [c]	15.3±0.1 [e]	237.2 ±2.4 [b]	90.21±1.0 [d]	4.5 ±0.06[de]	34.8 ±0.4 [e]	76.85±1.6 [c]
30% Meth	20.0 ±0.1 [d]	208.0 ±1.8 [f]	84.92±1.1 [d]	15.1 ±0.2 [e]	226.4 ±2.0 [c]	93.05±1.2 [c]	4.0 ±0.04 [e]	31.8 ±0.6 [f]	79.45±1.0 [b]
2.0M Acid, Meth	40.4±0.6 [a]	337.5 ±2.4 [c]	91.91±1.0 [b]	27.0 ±0.3 [a]	384.4 ±2.5 [a]	96.21±1.0 [b]	72.6 ±1.0 [a]	149.5 ±1.2 [c]	81.25±1.2 [b]
1.0M Acid.Meth	40.5±0.5 [a]	346.2 ±2.0 [b]	94.01±0.9 [a]	22.6±0.2 [b]	391.2 ±2.1 [a]	96.89±1.0 [b]	67.3 ±1.1 [b]	158.8 ±1.0 [b]	85.99±0.5 [a]
0.5M Acid.Meth	40.7±0.5 [a]	354.4 ±1.9 [a]	94.48±0.9 [a]	21.6±0.3 [c]	396.4 ±1.9 [a]	98.86±1.1 [a]	57.4 ±1.0 [c]	164.7 ±1.1 [a]	86.21±0.9 [a]

488 Values are mean ± SD of three replications. Solvents are, methanol (Meth) or acidified
489 methanol (Acid.Meth).
490 Different letters in each column represent significant differences ($p \leq 0.05$) among
491 solvents used.
492 [A] Yield % (w/w dry biomass).
493 [B]TPC, total phenolic contents expressed as mg gallic acid equivalents (GAE)/g extract.
494 [C] Value %age Scavenging by extract concentration 5 mg/mL.
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500 **Table 2:** LC-ESI-MS/MS characterization of 0.5M acidified methanol extracts from *Momordica*

501 *dioica (*Spiney gourd, Jungli karela*)*

Peak No.	RT(min)	MW	[M−H]⁻	MS² Ions	Identified Compounds	Molecular Formula**	Conc.* (ppm)	AAuth
5	9.5	______	161.1	89	Unknown	______	259.64	
2	3.06	______	333.32	273	Unknown	______	261.38	
4	4.83	316.22	315.11	301,257	Methyl ellagic acid	$C_{15}H_8O_8$	261.76	Y
3	4.23	434.4	433.11	271,153	Naringenin-7-O-glucoside	**$C_{21}H_{22}O_{10}$**	263.25	Y
6	21.84	270.24	269.04	227,197	Galangin	$C_{15}H_{10}O_5$	290.44	Y
9	23.79	______	303.07	285	Unknown	______	298.01	
8	23.43	302.24	300.98	179,151	Quercetin	$C_{15}H_{10}O_7$	313.73	
1	0.02	302.27	301.07	258,143	Hesperetin	$C_{16}H_{14}O_6$	320.71	Y
7	22.65	302.2	301.03	257	Ellagic acid	$C_{14}H_6O_8$	341.97	Y
10	24.27	360.31	359.12	161,197	Rosmarinic acid	$C_{18}H_{16}O_8$	358.85	Y
12	25.63	480.38	479.05	317,179	Myricetin-3-O-glucoside	**$C_{21}H_{20}O_{13}$**	378.65	Y
16	28.26	______	293.1	259	Unknown	______	407.76	
11	24.79	446.34	445.04	269, 175	Apigenin-7-O-glucuronide	**$C_{21}H_{18}O_{11}$**	411.83	Y
18	29.29	464.38	463.12	316	Myricitrin	**$C_{21}H_{20}O_{12}$**	423.19	
15	27.5	368.11	367.06	173, 191	4-Feruloyl-quinic acid	$C_{17}H_{20}O_9$	423.48	Y
14	27.1	318.24	317.08	179, 151	Myricetin	$C_{15}H_{10}O_8$	439.78	
13	26.46	312.23	311.07	149, 179, 135	Caftaric acid	**$C_{13}H_{12}O_9$**	460.16	Y
17	28.92	448.38	447.14	301, 179, 151	Quercitrin	**$C_{21}H_{20}O_{11}$**	532.07	Y
19	29.74	______	427.05	409	Unknown	______	573.11	

502 *Ranked by concentration (ppm), ** glycosides are shown in bold, Y = Peak authenticated by

503 published resources.

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507 Tasble 3. LC-ESI-MS/MS characterization of 0.5M acidified methanol extracts from
508 *Citrullus colocynthus L. (bitter cucumber, desert gourd, egusi,)*

Peak No.	RT(min)	MW	$[M-H]^-$	MS^2 Ions	Identified Compounds	Molecular Formula**	Conc.* (ppm)	Auth
7	6.18	______	327.25	309	Unknown	______	254.16	
1	2.31	168.14	167.14	152,123	Vanillic acid	$C_8H_8O_4$	254.86	Y
3	3.39	316.26	315	153, 09	Protocatechuic acid hexoside	$C_{13}H_{16}O_9$	254.89	Y
9	21.56	342.3	340.96	179, 35	Caffeoyl glucose	**$C_{15}H_{18}O_9$**	255.74	Y
10	22.7	432.38	431.07	269	Apigenin-7-glucoside	$C_{21}H_{20}O_{10}$	256.43	Y
2	2.96	176.12	175.01	115	Ascorbic acid	$C_6H_8O_6$	256.52	
8	10.37	270.24	268.97	119, 53, 243	Apigenin	$C_{15}H_{10}O_5$	256.87	Y
11	24.73	480.38	478.96	317, 79, 151	Myricetin 3-O-glucoside	**$C_{21}H_{20}O_{13}$**	259.05	Y
6	4.88	302.2	301.05	257	Ellagic acid	$C_{14}H_6O_8$	263.42	Y
12	25.52	270.28	268.98	197, 33	Alpinetin	$C_{16}H_{14}O_4$	264.59	Y
5	4.6	194.18	193.09	179, 49,134	Ferulic acid	$C_{10}H_{10}O_4$	266.3	
13	26.43	594.52	593.16	285,325	Kaempferol-3-rutinoside	**$C_{27}H_{30}O_{15}$**	279.37	Y
14	27.69	464.38	463.11	316	Myricitrin	$C_{21}H_{20}O_{12}$	311.69	Y
15	27.95	286.24	285.06	213,151, 133	Luteolin	$C_{15}H_{10}O_6$	329.74	Y
16	28.37	464.38	463.16	301	Isoquercitrin	**$C_{21}H_{20}O_{12}$**	392.62	Y
17	28.84	286.24	285.08	241,169,151	Kaempferol	$C_{15}H_{10}O_6$	394.08	
4	4.24	224.21	223.02	208, 179,164	Sinapic acid	$C_{11}H_{12}O_5$	409.51	
18	29.65	______	334.92	351	Unknown	______	409.8	
19	29.85	474.37	472.96	311, 179	Chicoric acid	$C_{22}H_{18}O_{12}$	454.92	

509 *Ranked by concentration (ppm), **phenolic glycosides are shown in bold, Y = Peak
510 authenticated by published resources.

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516 **Table 4.** LC-ESI-MS/MS characterization of 0.5M acidified methanol extracts from
517 *Cucumis melo var. agrestis (mouse melon, chibber, ucado melon*)

Peak No.	RT(min)	MW	[M−H]⁻	MS² Ions	Identified Compounds	Molecular Formula**	Conc* (ppm)	Auth
7	5.46	254.24	253.19	181,151, 101	Chrysin	$C_{15}H_{10}O_4$	257.48	Y
6	5.06	194.18	193.14	179,149, 134	Ferulic acid	$C_{10}H_{10}O_4$	258.06	Y
10	24.03	484.36	482.98	313,169	1,6-Di-O-galloyl glucoside	$C_{20}H_{20}O_{14}$	258.88	Y
5	4.62	______	288.06	244	Unknown	______	259.11	
3	3.36	184.15	183.16	169, 125	Methyl Gallate	$C_8H_8O_5$	259.75	Y
11	24.79	464.38	463.05	301	Isoquercetin	$C_{21}H_{20}O_{12}$	264.59	Y
8	22.26	342.3	340.92	179, 135	Caffeoyl glucose	**$C_{15}H_{18}O_9$**	269.8	Y
4	4.36	386.35	385.04	223	Sinapic acid hexoside	**$C_{17}H_{22}O_{10}$**	289.85	Y
2	0.87	198.17	197.1	182, 153	Syringic acid	$C_9H_{10}O_5$	290.15	
12	25.58	______	261.14	199	Unknown	______	334.98	
1	0.02	330.27	329.15	315, 165	Bis-methylated quercetin	$C_{17}H_{14}O_7$	343.71	Y
9	22.9	448.38	447.08	301, 79, 151	Quercitrin	**$C_{21}H_{20}O_{11}$**	344.29	Y
13	26.5	494.14	493.18	331	Malvidin-3-O-glucoside	**$C_{23}H_{26}O_{12}$**	399.61	Y
14	28.28	360.31	359.15	197	Glycosyringic acid	$C_{15}H_{20}O_{10}$	445.6	Y

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519 *Ranked by concentration (ppm), **phenolic glycosides are shown in bold, Y = Peak authenticated by
520 published resources.

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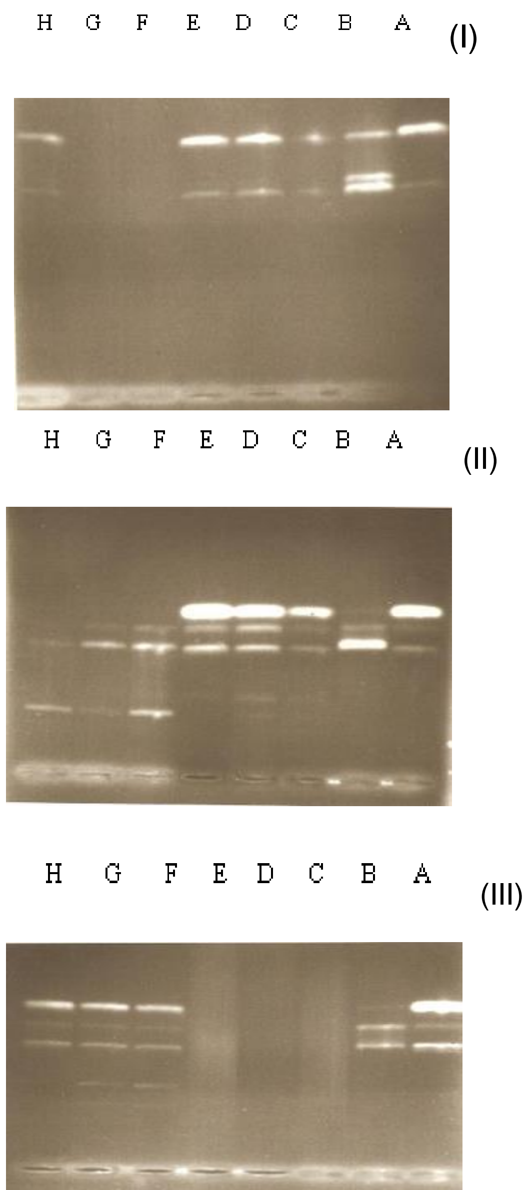
530 **List of Figures (1)**



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532 **Figure 1**: Electropherograms for DNA protection assay for oxidative stress using
533 extracts from (I) *Momordica dioica*, (II) *Citrullus colocynthus L.* and (III) *Cucumis melo*
534 *var. agrestis*. The direction of migration is upwards. For Figure I-III, lane A = untreated
535 DNA plasmid, lane B = DNA plasmid+ H_2O_2/UV treatment, lane C-E = lane B+ 0.5M
536 acidified methanol extract, Lane F-H = lane B +70% aqueous methanol extract.
537 Treatment concentration was 5, 10 and 20 mg/ml in lanes C-E, and F-H.

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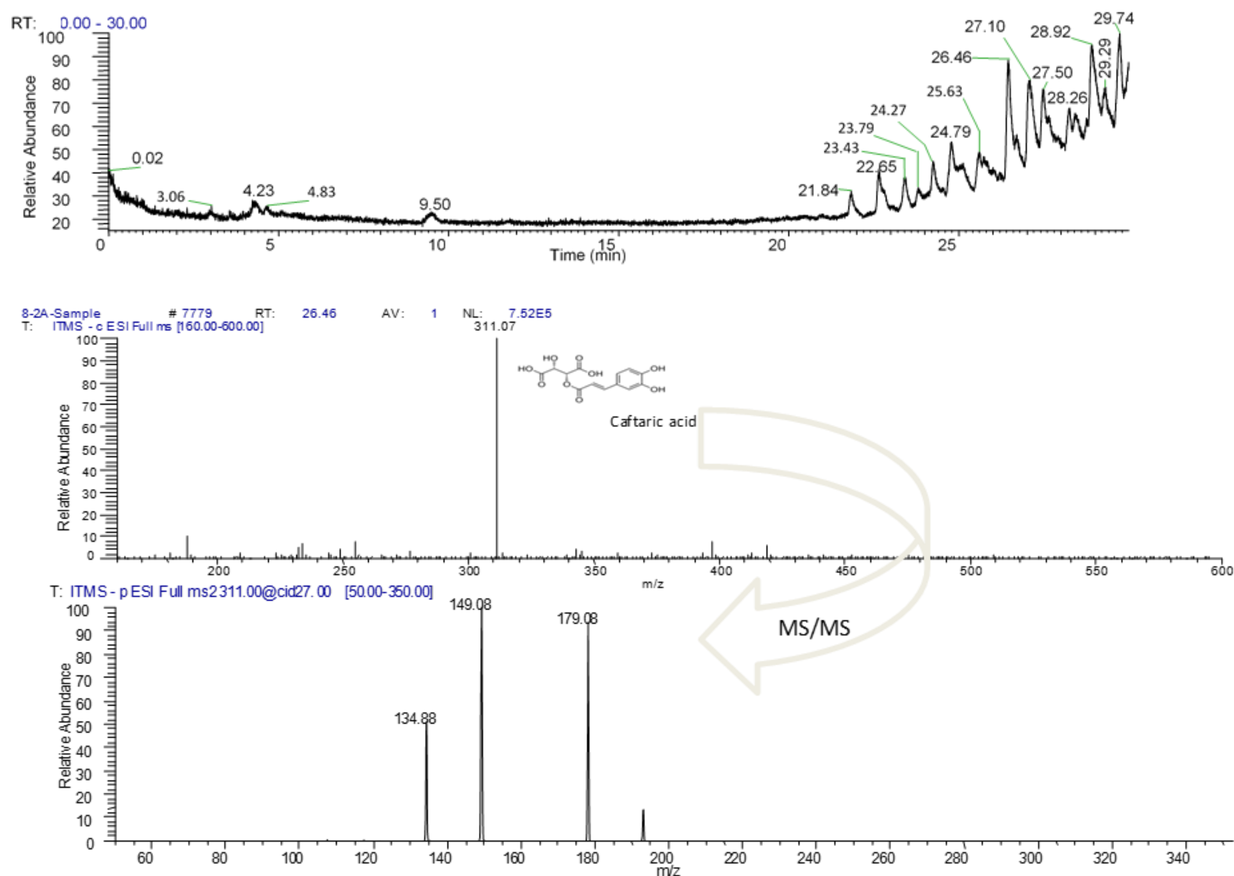

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542 Figure 2: Sample LC/MS/MS data for *Momordica dioca extract.* Peak with a retention

543 time 26.46 min was analyzed with an MS peak at m/z 311.07 indicating the presence of

544 Caftaric acid and confirmed by MS/MS fragments (see text for details).

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