**SUPPLEMENTARY MATERIAL**

**Online Antioxidant Activity and Ultra-performance LC-Electrospray Ionization-Quadrupole Time-of-ﬂight Mass Spectrometry for Chemical Fingerprinting of Indian Polyherbal Formulations**

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**Abstract**

A HPLC-DAD.DPPH method was developed for evaluating the 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity of ethylacetate extracts of different polyherbal formulations (draksarista, draksava, lohasava and arvindasava) by using RP-18e column. The ethylacetate extract from polyherbal, “draksarista” exhibited maximum free radical scavenging activity (99.9 ± 0.38%) followed by draksava (99.8 ± 0.34 %), lohasava (98.5 ± 0.30 %) and arvindasava (42.3 ± 0.34 %) at 100 µg mL-1. Simultaneously, ultra-performance liquid chromatography coupled with electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS) was used to study chemical composition of the ethylacetate extracts of formulations. The characteristic electrospray mass ionization reveals the dominance of polyphenols and their glycosides in the four polyherbal formulations.

**Keywords:** Draksarista; draksava; lohasava; antioxidant activity; ultra performance liquid chromatography

**Experimental**

***Material and Reagents***

The herbal formulations (draksarista, draksava, lohasava and arvindasava) were received from NIPER Institute in 2011. All HPLC grade solvents (methanol, acetonitrile and water) and trifluoroacetic acid (TFA) were obtained from Merck (Germany). 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) and L-ascorbic acid was purchased from Sigma–Aldrich (Delhi, India). Folin Ciocalteu’s reagent was purchased from S.D. Fine Chem Limted (Mumbai, India). Gallic acid was isolated and characterized in our laboratory.

***Sample Preparation***

The herbal formulations suffixed as “arishtas” are the preparations which are subjected to fermentation for an allotted period after boiling the main decoction and adding the other herbs. The formulations suffixed as “asavas" are the preparations which are subjected to fermentation for an allotted period with all its herbs mixed with certain quantity of water. The alcohol, thus, generated during fermentation helps in the extraction of bioactive molecules. The herbal formulations (500 mL each) were concentrated to dryness, re-dissolved in minimum amount of water and each formulation was partitioned with ethylacetate. The combined extracts of each formulation were distilled under vacuum at 45 ± 5 0C, dried in lyophilizer and stored at 4 0C until use.

*Total Phenolic Content*

Total phenolic content in extracts was determined according to the Folin-Ciocalteu procedure. Appropriately diluted extract (0.2 mL) was mixed with 1.0 mL of Folin-Ciocalteu reagent (1:10, v/v diluted with water) and incubated for 1 min before 0.8 mL sodium carbonate (7.5% w/v) was added. The mixture was incubated for 2 hours at room temperature before absorbance was measured at 750 nm against deionized water blank using ELISA plate reader (Labsystems, Helsinki, Finland). Total phenolic content was estimated from a standard curve of gallic acid and were expressed as milligrams of gallic acid equivalent (GAE) per gram.

***DPPH Scavenging Assay***

The free-radical-scavenging capacity of extracts was evaluated with the DPPH stable radical following the methodology described by Blios (1958). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 2 mL of this solution was added to 0.3 mL of different extract concentrations (5–100 µg mL-1) and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm against the blank, which did not contain the extract. The L-ascorbic acid was used as the positive control.

***HPLC-DAD-DPPH analysis***

HPLC analysis was performed on waters HPLC system equipped with photo diode array detector (PDA). The chromatography was performed on RP-18e column (250 × 4.6 mm, 5 µm particle size) at a column temperature 27 0C using methanol : water (80 : 20) as mobile phase in isocratic elution with flow rate of 1 ml min-1. The DPPH peaks were monitored at 517 nm. The difference in the reduction of peak area of DPPH (PA) between blank and the sample was used for determining the percent of free radical scavenging activity of the sample. The radical-scavenging activity (percent inhibition) was expressed as percentage of DPPH radical elimination calculated according to the following equation:

Percent inhibition (%) = (Acontrol \_ Asample/Acontrol) **×**100

Where Acontrol is the absorbance of the positive control and Asample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average value was calculated.

The change in absorbance intensity because of DPPH turning yellow was plotted against the sample concentration in order to calculate the IC50 value, which is the amount of sample necessary to decrease the absorbance of DPPH by 50%.

***UPLC-electrospray ionization-quadrupole time-of-flight mass spectrometry***

UPLC was performed using a Waters ACQUITY UPLC System (Waters, Milford MA, USA). Separation was achieved using an ACQUITY UPLC® BEH C18 column (100 mm, 2.1 mm i.d., 1.7 µm particle size; Waters) maintained at 30 0C, with a mobile phase flow rate of 0.30 mL/ min. The system operating pressure was 8200 psi at initial gradient conditions. The mobile phase contained water, 0.05% trifluoroacetic acid (A) and acetonitrile (B). Gradient elution was employed starting at 20% B, held for 0.20 min, then shifting linearly B to 45% over 1.40 min, 55% over 1.70 min, 65% over 2.20 min, 35% over 3.50 min and re-equilibrated for 1.0 min, giving a total cycle time of 4.5 min. The injection volume was 10 µL with partial loop injection using needle overfill mode. The peaks were detected at 280 nm. A time-of-flight mass spectrometer with electrospray ionization (ESI-MS) interface was used for fingerprinting (Micromass, Manchester, UK). For UPLC analysis, data acquisition was performed using positive ion mode over a mass range of *m/z* 50–1000. The general conditions were: source temperature of 80 0C, capillary voltage of 2.1 kV and cone voltage of 23 V. MassLynx 4.1 (Waters, Manchester, UK) software was used for data analysis.

***Tandem mass spectrometry***

Structural analysis of single molecular ion in the mass spectra from extracts was performed by mass-selecting the ion of interest, which was in turn submitted to 15–35 eV collisions with argon in the collision quadruple.

**Table captions:**

**Table S1.**

Identification of phenolic compounds/markers in different polyherbal formulations

**Table S2.**

Antioxidant activity and total phenolics of ethylacetate extracts of polyherbal formulations

**Table S1.** Identification of phenolic compounds/markers in different polyherbal formulations

**“Draksarista”**

**Plants used :** *Vitis* *vinifera*, *Cinnamomum zeylanicum*, *Elettaria cardamomun*, *Cinnamomun tamala*, *Mesua ferrea*, *Piper longum*, *Piper nigrum*, *Emblica ribes*, *Woodfordia fruticosa*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak**  **No.** | **tR** | **UV spectra** | **MW** | **Positive ion mode**  **[M+H]+/Na** | **Identified compounds** |
| 1 | 1.40 | 215, 271 | 310, 332 | 311, 333 | Galloyl ester, Monogalloylglucoside |
| 2 | 1.80 | 277, 297sh | 330 | 331 | Malvidin |
| 3 | 2.64 | 255, 360 | 476 | 477 | Ellagic acid-acetylpentoside |
| 4 | 2.72 | 255, 360 | 434 | 435 | Ellagic acid-pentoside |
| 5 | 2.94 | 264, 290sh | 462 | 463 | Protocatechuic glucosyl rhamnoside |
| 6 | 3.38 | 253, 355 | 470 | 471 | Ellagic acid derivative |
| 7 | 3.54 | 279 | 446 | 447 | Procyanidin dimer |
| 8 | 3.61 | 289, 322sh | 476 | 477 | Naringenin-acetylglucoside |
| 9 | 3.68 | 217, 273 | 608 | 609 | Peonidin-3-(6´´-coumaroyl glucoside) |
| 10 | 4.09 | 278 | - | - | Procyanidin dimer |
| 11 | 4.35 | 255, 360 | - | 449 | Quercetin rhamnoside |

**“Draksava”**

**Plants used :** *Withania somnifera*, *Argyreia speciosa*, *Pueraria tuberosa*, *Eugenia caryophyllata*, *Croton tiglium*, *Piper cubeba*, *Piper chaba, Vitis* *vinifera*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 1 | 1.40 | 215, 271 | 310, 332 | 311, 333 | Galloyl ester, Monogalloylglucoside |
| 2 | 1.80 | 277, 297sh | 331 | 331 | Malvidin |
| 3 | 2.64 | 256, 360 | 477 | 477 | Ellagic acid-acetylpentoside |
| 4 | 2.72 | 255,360 | 434 | 435 | Ellagic acid-pentoside |
| 5 | 2.94 | 266, 290sh | 462 | 463 | Protocatechuic glucosyl rhamnoside |
| 6 | 3.38 | 255, 355 | 470 | 471 | Ellagic acid derivative |
| 7 | 3.54 | 279 | 446 | 447 | Procyanidin dimer |
| 8 | 3.61 | 289, 320sh | 476 | 477 | Naringenin-acetylglucoside |
| 9 | 3.68 | 216, 274 | 608 | 609 | Peonidin-3-(6´´-coumaroyl glucoside) |
| 10 | 4.09 | 278 | - | - | Procyanidin dimer |
| 11 | 4.35 | 254, 360 | - | 449 | Quercetin rhamnoside |

**“Lohasava”**

**Plants used :** *Solanum xanthocarpum,**Zingiber officinale*, *Piper longum*, *Piper nigrum*, *Emblica ribes*, *Terminalia chebula*, *Terminalia belerica*, *Embelia ribes*, *Cyprus rotundus*, *Plumbago zeylanica*, *Woodfordia fruticosa*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 1 | 1.40 | 215, 271 | 332 | 333 | Monogalloylglucoside |
| 2 | 1.80 | 280, 295sh | 330 | 331 | Malvidin |
| 3 | 2.40 | 250, 290 | 432 | 433 | Protocatechuic acid derivative |
| 4 | 2.72 | 254, 360 | 434 | 435 | Ellagic acid-pentoside |
| 5 | 2.94 | 263, 290sh | 462 | 463 | Protocatechuic glucosyl rhamnoside |
| 6 | 3.38 | 253,355 | 470 | 471 | Ellagic acid derivative |
| 7 | 3.54 | 279 | 446 | 447 | Procyanidin dimer |
| 8 | 3.68 | 216, 271 | 608 | 609 | Peonidin-3-*O*-glucoside |
| 9 | 4.09 | 278 | - | 431 | Procyanidin dimer |
| 10 | 4.32 | 280 | - | 481 | Unknown galloyltannina |

a Characterized on the basis of UV spectrum

**“Arvindasava”**

**Plants used :** *Terminalia arjuna*, *Nelumbo nucifera*, *Vetiveria zizanioidies*, *Gmelina arborea*, *Nymphaea stellata*, *Rubia cordifolia*, *Elettaria cardamomum*, *Sida cordifolia*, *Nardostachys jatamansi*, *Cyprus rotundus*, *Emblica officinalis*, *Emblica ribes*, *Acorus calamus*, *Hedychium spicatum* , *Vitis vinifera*, *Glycyrrhiza glabra*, *Madhuca indica*, *Terminalia arjuna*, *Fumaria parviflora*, *Indigofera tinctoria*, *Cryptolepis buchanani*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 1 | 1.40 | 212, 270 | 332, 346 | 333, 347 | Monogalloylglucoside, Methylgallate glucopyranoside |
| 2 | 1.80 | 283, 295 | 330 | 331 | Malvidin |
| 3 | 2.40 | 250, 290 | 432 | 433 | Protocatechuic acid derivative |
| 4 | 2.72 | 253, 360 | 434 | 435 | Ellagic acid-pentoside |
| 5 | 2.94 | 261, 295sh | 462 | 463 | Protocatechuic glucosyl rhamnoside |
| 6 | 3.04 | 220, 279 | 290 | 291 | Catechin/epicatechin |
| 7 | 3.54 | 279 | 446 | 447 | Procyanidin dimer |
| 8 | 4.09 | 278 | - | 431 | Uncharacterized |
| 9 | 4.32 | 278 | - | 481 | Unknown galloyltannina |

**Table S2.** Antioxidant activity and total phenolics of ethylacetate extracts of polyherbal formulations

|  |  |  |  |
| --- | --- | --- | --- |
| Standard /Extract | Antioxidant activity  DPPH scavenging effect (%) DPPH (IC50)  at 100 µg/mL (µg/mL) | | Total phenolic content  (mg /g of GAE) |
| L-Ascorbic acid | 99.9 ± 0.28 | 20.8 ± 0.32 |  |
| Draksarista | 99.9 ± 0.38 | 12.7 ± 0.28 | 235.16 ± 0.16 |
| Draksava | 99.8 ± 0.34 | 20.0 ± 0.25 | 218.83 ± 0.21 |
| Lohasava | 98.5 ± 0.30 | 21.7 ± 0.36 | 201.33 ± 0.14 |
| Arvindasava | 42.3 ± 0.34 | - | 22.16 ± 0.18 |

**Figure captions:**

**Figure S1.**

HPLC chromatogram of DPPH (a) blank , (b) after incubation with polyherbal formulation.

**Figure S2.**

Free–radical–scvanging activity of ethylacetate extracts and L-ascorbic acid (positive control) by HPLC measured at *λ*max517 nm using the DPPH assay.

**Figure S3.**

Chemical structures of the identified compounds.

**Figure S4.**

UPLC-DAD chromatograms of ethylacetate extracts at *λ*max 280 nm from (a) draksarista, (b) draksava, (c) lohasava and (d) arvindasava.

**Figure S5.**

ESI-(+)-MS of compounds observed in the polyherbal formulations.



**(a)**

**UV 517 nm**

**UV 517 nm**

**(b)**

Figure. S1. HPLC chromatogram of DPPH (a) blank , (b) after incubation with polyherbal formulation

Figure. S2. Free–radical–scvanging activity of ethylacetate extracts and L-ascorbic acid (positive control) by HPLC measured at *λ*max517 nm using the DPPH assay.



Figure S3. Structures of the compounds observed in herbal formulations.



**a**



**c**

**b**





**d**

Figure. S4. UPLC-DAD chromatograms of ethylacetate extracts at *λ*max 280 nm from (a) draksarista, (b) draksava, (c) lohasava and (d) arvindasava.



**a**

**b**

**c**

**d**







**e**



**f**



**j**

**g**

**h**

**i**







Figure. S5. (a) Galloyl ester, (b) malvidin, (c) ellagic acid-acetylpentoside, (d) ellagic acid pentoside, (e) protocatechuic glycosyl rhamnoside, (f) ellagic acid derivative, (g) procyanidin dimer (h) peonidin-3-(6’’-coumaroyl glycoside), (i) quercetin rhamnoside, (j) catechin/epicatechin.