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ORIGINAL ARTICLE

Chromatographic method development and metabolite profiling for biomass and extraction optimization of withametelin and daturaolone from *D. Innoxia* Mill.

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Seasonal variation

Abstract Low yields of isolated natural compounds halt the drug discovery process as they can only be used for structure elucidation studies and basic biological screening. Metabolite profiling *via* chromatographic means for optimized selection of biomass and extraction medium can help resolve the issue. In line with this, the project is focused on metabolite profiling of *Datura innoxia* regarding its two bioactive principals i.e., withametelin and daturaolone. Samples (840) were prepared *via* collection of five parts (leaves, stem, fruit, root, flowers) from two geographically different regions of Pakistan i.e., Islamabad and Muzaffargarh for six months (May-October) and extraction in fourteen solvent systems of varied polarity range, respectively. Six months agroclimatology data (temperature, humidity, soil wetness, UV irradiance) was also obtained. TLC co-detection method (n-hexane: ethyl acetate; 7:3) of withametelin and daturaolone was developed and analysis was performed on all samples. RP HPLC method was developed for withametelin (Linearity = R^2;0.9) and daturaolone (linearity: R^2;0.9) and 118 samples which showed detections in TLC analysis were quantified. Withametelin was mostly detected in leaves with a maximum quantified value of 5.12 ± 0.28 µg/mg dry plant powder when collected in June from arid Muzaffargarh region and extracted with Ethyl acetate + Ethanol (1:1). Distribution of daturaolone is mostly found in fruits with a maximum quantified value of 5.18 ± 0.45 µg/mg dry plant powder when collected in August from mountainous Islamabad region and extracted with Ethyl acetate + Ethanol (1:1). The study states that the presence and quantitative variations of withametelin and daturaolone depend on the

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plant's part, extraction medium, geographical location, weather conditions and soil wetness. Use of a controlled environment research to determine the quantitative relationship between different parameters is proposed.

1. Introduction

Metabolite profiles are the analysis of specific metabolic pathways or compounds associated with the pathways. It is more specific than the metabolite fingerprint and follows specific hypotheses (Wolfender et al., 2009). Therefore, distinctive analytical methods for determining the analytes are utilized. The method is the oldest, most established and a pioneer of metabolomics. Some reports estimate that there are up to 15,000 different compounds in a particular plant species. More than 200,000 natural compounds have been reported so far. By assessing the chemical space of natural products, it is possible to quantify and visualize wide range of natural constituents. The chemical diversity of natural compounds is directly related to the high variability of the physical and chemical properties of the natural product, making it very difficult to distinguish, detect, and identify natural matrices. Therefore, single analytical technique is not sufficient to analyze complex metabolomes in their entirety, and multiple technologies are necessary (Wolfender et al., 2015).

Finding practical ways to strengthen the process and increase yields of selected metabolite is a major challenge for researchers. Compounds are associated with environmental adaptation and play an important biological role. Until now, there have been many studies on the search for the highest yield of desired metabolites and the optimization of cultural conditions. However, few studies directly stressed the adaptability of secondary metabolites to environmental disturbances. Environmental and ecosystem conditions, geographical areas, collection seasons, harvest times, genotypes, and ecological types influence quantitative and qualitative composition. Therefore, plant secondary metabolism is seen as a plant behaviour, which is part of the ability to adapt and survive to environmental stimuli throughout its lifetime. In pharmaceutical plants, environmental conditions can redirect metabolism, thus regulating the production of active compounds (Yang et al., 2018).

In our previous studies, withametelin and daturaolone were isolated from *Datura innoxia* Mill. which possess drug like features and good pharmacokinetic profiles, respectively (Baig et al., 2020, Baig et al., 2021). Their perceived molecular targets are considered to play an important role in inflammation, pain, brain disease, and cancer. They showed significant cytotoxicity in cancer cell lines and protein kinase inhibition. In addition, analgesics, anti-inflammatory and antidepressant effects from acute in vivo analysis have also been observed. Both natural compounds are proposed for their detailed mechanistic, toxicity profile, and clinical studies. However, low yield is a halt in drug discovery because mostly isolated bioactive compounds are available for detection or for basic screening only and the process to isolate them is not replicative. Consequence is the lack of detailed pharmacological evaluation. Therefore, development of a standardized method not only helps in detection of bioactive compounds but also in selection of an optimized herbal source for large-scale isolation. In line with this, the current project is focused on metabolic profiling of *Datura innoxia* with reference to its two bioactive principles i.e. withametelin and daturaolone. Discovering chemical compounds from natural sources sounds scientifically interesting, but optimized biomass selection and yield augmentation for thorough pharmacological role determination are the actual challenges to acquire ultimate benefits. To the best of our knowledge, no study has been presented so far which describes the chromatography based detection and quantification study to determine the best plant part, geographical area, solvent system and climatic conditions for optimized biomass selection to obtain withametelin and daturaolone.

2. Methodology

2.1. Selection of sites and collection of samples

The sample location was chosen to signify the growing area of *D. innoxia* and showed a significant change in the edaphic and climatic factors affecting the growth of respective plant specie. Accordingly, *D. innoxia* was collected from two geographically different sites in Pakistan namely Islamabad (I) and Muzaffargarh (Mz). The sampling was carried out in two cities within a radius of 500 m over a period of six months (May to October). The selected (uniform) plants in the fruiting and flowering phase were sampled in order to do the collection on the same date of the month (15th). Each sample of the plant was placed in a plastic bag with appropriate labeling. Samples were returned to the laboratory within 24 h of field collection.

2.2. Weather parameter record collection

The detailed agroclimatology reports of 1 year (January 2018 to December 2018) of selected sites were downloaded from the authenticated source in CSV format and 6 month agroclimatology data was utilized in the current project. The data was obtained from the National Aeronautics and Space Administration (NASA) Langley Research Center (LaRC) Prediction of Worldwide Energy Resource (POWER) Project funded through the NASA Earth Science/Applied Science Program (NASA, 2022) (https://power.larc.nasa.gov/).

2.3. Sample preparation

The collected plant was washed under tap water. Leaves (L), stem (S), roots (R), flowers (Fl), and fruits (Fr) were separated, and shade dried for up to 3 weeks. Samples were grounded to a fine powder. Pre-weighed (50 mg) each dried plant part in Eppendorf tubes was macerated (1 mL) in varied polarity solvents systems either alone or in 1:1 combination. Solvent systems and their combination include; n-hexane (nH), chloroform (C), acetone (A), ethyl acetate (Ea), methanol (M), ethanol (E) water (W), nH + C, nH + Ea, C + Ea, C + M, A + Ea, Ea + M and Ea + E. Occasional shaking and ultrasonication aided maceration were done for 3 days. Ultrasonication was performed thrice daily for 5 min each at a frequency of 40 kHz. Each sample was centrifuged, and the supernatant was separated. Solvent system and their combinations are given in Table 1. In brief, 5 plant parts were collected from two geographical locations for six months. Each

plant part was macerated in 14 solvent systems respectively. A total of 840 samples were prepared for the TLC analysis.

2 *locations* ∗ 6 *months* ∗ 5 *parts* ∗ 14 *solvent systems*

= 840 *samples*

Appropriate coding of each sample is given in Table 1.

Daturaolone and withametelin were isolated and purified in our previous work. Daturaolone (1 mg/ml) solution was prepared in chloroform. Withametelin (1 mg/ml) solution was prepared in ethyl acetate. 500 µl of the corresponding solution were mixed for co-detection and analysis on TLC plates. The final concentration of respective compounds was 0.5 µg/µl.

2.4. TLC detection method optimization and sample analysis

Normal phase TLC plates were used. Firstly, TLC method was optimized for the co-detection of withametelin and daturaolone. 1 µl of the standard solution was run in different mobile phases to select the best mobile phase for separation, elution and simultaneous detection of withametelin and daturaolone. Phosphomolybdic acid reagent was used for the final detection and analysis. After finalizing the TLC optimization of standards, samples were analyzed on 4 * 6.66 cm TLC plates. 1 µl of each plant sample was spotted on TLC plate and elution was done. TLC plate number, sample serial number, coding and sequence in which each sample was spotted on TLC plate along with the standard solution are given in Table 1. Each TLC analysis was performed in triplicate. Plant samples that gave detection of withametelin and daturaolone were selected for HPLC detection and quantification.

2.5. RP HPLC method development

2.5.1. Instrumentation and analytical conditions

The analysis of the study was carried out on the HPLC Agilent 1200 series system. The tests were conducted on the C8 column with a dimension of 4.6x250 mm, a size of 5 µm of silica, and a mixture of mobile phase composition. A gradient mobile phase system was used with mobile phase A (Methanol: Water 1:1) and mobile phase B (100% methanol). The flow rate was adjusted to be 1 mL/min throughout the experiment. The injection volume was 50 µl. Gradient percent mobile phase B at different time intervals include: 0% at 0 min, 100 % at 10 min to 18 min and 0% at 19 to 25 min. The selected wavelengths for quantitative analysis were 230 nm for withametelin and 210 nm for daturaolone. Stop time was 25 min.

2.5.2. Preparation of solutions

The stock solutions of withametelin and daturaolone were prepared by dissolving them in methanol. Solutions were protected from light and were stored at 4 °C. Calibration curve was generated by analysis at final concentrations of 0.31–10 µg/ml.

2.5.3. Linearity

Linearity was determined by three injections of withametilin and daturaolone at two-fold serial concentrations (0.31–10 µg/ml). The peak area was plotted against concentrations. Then, linearity was evaluated using calibration equations to calculate correlation coefficients, slope coefficients, and intercept. Correlation coefficient (R) greater than 0.98, was considered acceptable (Table 2) (Guideline, 2005, Landim et al., 2013).

2.5.4. Sensitivity

The detection (LOD) and quantification LOQ) limits were determined by the calibration curves of the withametelin and daturaolone standards. According to the ICH guidelines, LOD is calculated according to the expression DPx3.3/ IC, where DP is the standard deviation of the response and IC is the slope of the calibration curve. LOQ was created with the help of the expression DP x10/IC (Table 2) (Guideline, 2005, Landim et al., 2013, Seo et al., 2016).

2.5.5. Accuracy

The accuracy was evaluated through recovery assays carried out by adding known amounts of standards withametelin (0.5, 1 and 1.5 µg/mL) and daturaolone (0.7, 1.4 and 2.1 µg/mL) to the sample. Each solution was injected three times (Guideline, 2005, Landim et al., 2013, Seo et al., 2016).

2.5.6. Precision

To evaluate the intra-day precision of this method, the sample is injected three times a day. The inter-day precision was determined by the samples examined on different days, as well as by another analyst (Guideline, 2005, Landim et al., 2013, Seo et al., 2016).

2.5.7. Robustness

Three sample solutions of withametelin and daturaolone had been prepared and analyzed under established conditions but changing the wavelength parameter from 210 nm to 212 nm for daturaolone and 230 to 232 nm for withametelin and by varying the pH (0.2%) of the mobile phase (Guideline, 2005, Seo et al., 2016). Robustness was also checked by changing the column supplier (Landim et al., 2013).

2.5.8. RPHPLC sample preparation and quantification analysis

Samples that gave detection of withametelin and daturaolone in TLC analysis were used (Table 5). Previously separated supernatants were dried and resuspended in methanol to be used for the HPLC analysis. All the results were expressed as means ± standard deviation (SD) of three replicates.

2.6. Statistical analysis

Microsoft EXCEL 365 was used for statistical analysis. Graph Pad PRISM 5 was used for correlation analysis.

3. Results and discussion

3.1. Area and time-dependent agroclimatology data variations were observed

A six-month period agroclimatic research has been carried out. The agroclimatic parameters differ between the two sites of Islamabad and Muzaffargarh (Fig. 1). The average surface temperature of the earth, and the average air temperature (dry bulbs) at 2 m in the six months were highest in Muzaffargarh in June while lowest in Islamabad in October. Withame-

Table 1 Blueprint of samples (TLC number, sample number, plant part, extraction solvent, area, month) spotted on TLC plates.

D. Innoxia													
Leaves													
TLC 1 (nH)		**TLC 2 (C)**		**TLC 3 (A)**		**TLC 4 (Ea)**		**TLC 5 (M)**		**TLC 6 (E)**		**TLC 7 (W)**	
1	LnHIMay	**13**	LCIMay	**25**	LAIMay	**37**	LEaIMay	**49**	LMIMay	**61**	LEIMay	**73**	LWIMay
2	LnHIJune	**14**	LCIJune	**26**	LAIJune	**38**	LEaIJune	**50**	LMIJune	**62**	LEIJune	**74**	LWIJune
3	LnHIJuly	**15**	LCIJuly	**27**	LAIJuly	**39**	LEaIJuly	**51**	LMIJuly	**63**	LEIJuly	**75**	LWIJuly
4	LnHIAug	**16**	LCIAug	**28**	LAIAug	**40**	LEaIAug	**52**	LMIAug	**64**	LEIAug	**76**	LWIAug
5	LnHISep	**17**	LCISep	**29**	LAISep	**41**	LEaISep	**53**	LMISep	**65**	LEISep	**77**	LWISep
6	LnHIOct	**18**	LCIOct	**30**	LAIOct	**42**	LEaIOct	**54**	LMIOct	**66**	LEIOct	**78**	LWIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
7	LnHMzMay	**19**	LCMzMay	**31**	LAMzMay	**43**	LEaMzMay	**55**	LMMzMay	**67**	LEMzMay	**79**	LWMzMay
8	LnHMzJune	**20**	LCMzJune	**32**	LAMzJune	**44**	LEaMzJune	**56**	LMMzJune	**68**	LEMzJune	**80**	LWMzJune
9	LnHMzJuly	**21**	LCMzJuly	**33**	LAMzJuly	**45**	LEaMzJuly	**57**	LMMzJuly	**69**	LEMzJuly	**81**	LWMzJuly
10	LnHMzAug	**22**	LCMzAug	**34**	LAMzAug	**46**	LEaMzAug	**58**	LMMzAug	**70**	LEMzAug	**82**	LWMzAug
11	LnHMzSep	**23**	LCMzSep	**35**	LAMzSep	**47**	LEAMzSep	**59**	LMMzSep	**71**	LEMzSep	**83**	LWMzSep
12	LnHMzOct	**24**	LCMzOct	**36**	LAMzOct	**48**	LEaMzOct	**60**	LMMzOct	**72**	LEMzOct	**84**	LWMzOct
TLC 8 (nH + C)		**TLC 9 (nH + Ea)**		**TLC 10 (C + Ea)**		**TLC 11 (C + M)**		**TLC 12 (A + Ea)**		**TLC 13 (Ea + M)**		**TLC 14 (Ea + E)**	
85	LnH + CIMay	**97**	LnH + EaIMay	**109**	LC + EaIMay	**121**	LC + MIMay	**133**	LA + EaIMay	**145**	LEa + MIMay	**157**	LEa + EIMay
86	LnH + CIJune	**98**	LnH + EaIJune	**110**	LC + EaIJune	**122**	LC + MIJune	**134**	LA + EaIJune	**146**	LEa + MIJune	**158**	LEa + EIJune
87	LnH + CIJuly	**99**	LnH + EaIJuly	**111**	LC + EaIJuly	**123**	LC + MIJuly	**135**	LA + EaIJuly	**147**	LEa + MIJuly	**159**	LEa + EIJuly
88	LnH + CIAug	**100**	LnH + EaIAug	**112**	LC + EaIAug	**124**	LC + MIAug	**136**	LA + EaIAug	**148**	LEa + MIAug	**160**	LEa + EIAug
89	LnH + CISep	**101**	LnH + EaISep	**113**	LC + EaISep	**125**	LC + MISep	**137**	LA + EaISep	**149**	LEa + MISep	**161**	LEa + EISep
90	LnH + CIOct	**102**	LnH + EaIOct	**114**	LC + EaIOct	**126**	LC + MIOct	**138**	LA + EaIOct	**150**	LEa + MIOct	**162**	LEa + EIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
91	LnH + CMzMay	**103**	LnH + EaMzMay	**115**	LC + EaMzMay	**127**	LC + MMzMay	**139**	LA + EaMzMay	**151**	LEa + MMzMay	**163**	LEa + EMzMay
92	LnH + CMzJune	**104**	LnH + EaMzJune	**116**	LC + EaMzJune	**128**	LC + MMzJune	**140**	LA + EaMzJune	**152**	LEa + MMzJune	**164**	LEa + EMzJune
93	LnH + CMzJuly	**105**	LnH + EaMzJuly	**117**	LC + EaMzJuly	**129**	LC + MMzJuly	**141**	LA + EaMzJuly	**153**	LEa + MMzJuly	**165**	LEa + EMzJuly
94	LnH + CMzAug	**106**	LnH + EaMzAug	**118**	LC + EaMzAug	**130**	LC + MMzAug	**142**	LA + EaMzAug	**154**	LEa + MMzAug	**166**	LEa + EMzAug
95	LnH + CMzSep	**107**	LnH + EaMzSep	**119**	LC + EaMzSep	**131**	LC + MMzSep	**143**	LA + EaMzSep	**155**	LEa + MMzSep	**167**	LEa + EMzSep
96	LnH + CMzOct	**108**	LnH + EaMzOct	**120**	LC + EaMzOct	**132**	LC + MMzOct	**144**	LA + EaMzOct	**156**	LEa + MMzOct	**168**	LEa + EMzOct
						Stem							
TLC 15 (nH)		**TLC 16 (C)**		**TLC 17 (A)**		**TLC 18 (Ea)**		**TLC 19 (M)**		**TLC 20 (E)**		**TLC 21 (W)**	
169	SnHIMay	**181**	SCIMay	**193**	SAIMay	**205**	SEaIMay	**217**	SMIMay	**229**	SEIMay	**241**	SWIMay
170	SnHIJune	**182**	SCIJune	**194**	SAIJune	**206**	SEaIJune	**218**	SMIJune	**230**	SEIJune	**242**	SWIJune
171	SnHIJuly	**183**	SCIJuly	**195**	SAIJuly	**207**	SEaIJuly	**219**	SMIJuly	**231**	SEIJuly	**243**	SWIJuly
172	SnHIAug	**184**	SCIAug	**196**	SAIAug	**208**	SEaIAug	**220**	SMIAug	**232**	SEIAug	**244**	SWIAug
173	SnHISep	**185**	SCISep	**197**	SAISep	**209**	SEaISep	**221**	SMISep	**233**	SEISep	**245**	SWISep
174	SnHIOct	**186**	SCIOct	**198**	SAIOct	**210**	SEaIOct	**222**	SMIOct	**234**	SEIOct	**246**	SWIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
175	SnHMzMay	**187**	SCMzMay	**199**	SAMzMay	**211**	**SEaMzMay**	**223**	SMMzMay	**235**	SEMzMay	**247**	SWMzMay
176	SnHMzJune	**188**	SCMzJune	**200**	SAMzJune	**212**	SEaMzJune	**224**	SMzIJune	**236**	SEMzJune	248	SWMzJune
177	SnHMzJuly	**189**	SCMzJuly	**201**	SAMzJuly	**213**	SEaMzJuly	**225**	SMzIJuly	**237**	SEMzJuly	249	SWMzJuly
178	SnHMzAug	**190**	SCMzAug	**202**	SAMzAug	**214**	SEaMzAug	**226**	SMzIAug	**238**	SEMzAug	250	SWMzAug
179	SnHMzSep	**191**	SCMzSep	**203**	SAMzSep	**215**	SEAMzSep	**227**	SMzISep	**239**	SEMzSep	251	SWMzSep
180	SnHMzOct	**192**	SCMzOct	**204**	SAMzOct	**216**	SEaMzOct	**228**	SMzIOct	**240**	SEMzOct	252	SWMzOct

Table 1 (*continued*)

D. Innoxia													
Leaves													
TLC 1 (nH)		**TLC 2 (C)**		**TLC 3 (A)**		**TLC 4 (Ea)**		**TLC 5 (M)**		**TLC 6 (E)**		**TLC 7 (W)**	
TLC 22 (nH + C)		**TLC 23 (nH + Ea)**		**TLC 24 (C + Ea)**		**TLC 25 (C + M)**		**TLC 26 (A + Ea)**		**TLC 27 (Ea + M)**		**TLC 28 (Ea + E)**	
253	SnH + CIMay	**265**	SnH + EaIMay	**277**	SC + EaIMay	**289**	SC + MIMay	**301**	SA + EaIMay	**313**	SEa + MIMay	325	SEa + EIMay
254	SnH + CIJune	**266**	SnH + EaIJune	**278**	SC + EaIJune	**290**	SC + MIJune	**302**	SA + EaIJune	**314**	SEa + MIJune	326	SEa + EIJune
255	SnH + CIJuly	**267**	SnH + EaIJuly	**279**	SC + EaIJuly	**291**	SC + MIJuly	**303**	SA + EaIJuly	**315**	SEa + MIJuly	327	SEa + EIJuly
256	SnH + CIAug	**268**	SnH + EaIAug	**280**	SC + EaIAug	**292**	SC + MIAug	**304**	SA + EaIAug	**316**	SEa + MIAug	328	SEa + EIAug
257	SnH + CISep	**269**	SnH + EaISep	**281**	SC + EaISep	**293**	SC + MISep	**305**	SA + EaISep	**317**	SEa + MISep	329	SEa + EISep
258	SnH + CIOct	**270**	SnH + EaIOct	**282**	SC + EaIOct	**294**	SC + MIOct	**306**	SA + EaIOct	**318**	SEa + MIOct	330	SEa + EIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
259	SnH + CMzMay	**271**	SnH + EaMzMay	**283**	SC + EaMzMay	**295**	SC + MMzMay	**307**	SA + EaMzMay	**319**	SEa + MMzMay	331	SEa + EMzMay
260	SnH + CMzJune	**272**	SnH + EaMzJune	**284**	SC + EaMzJune	**296**	SC + MMzJune	**308**	SA + EaMzJune	**320**	SEa + MMzJune	332	SEa + EMzJune
261	SnH + CMzJuly	**273**	SnH + EaMzJuly	**285**	SC + EaMzJuly	**297**	SC + MMzJuly	**309**	SA + EaMzJuly	**321**	SEa + MMzJuly	333	SEa + EMzJuly
262	SnH + CMzAug	**274**	SnH + EaMzAug	**286**	SC + EaMzAug	**298**	SC + MMzAug	**310**	SA + EaMzAug	**322**	SEa + MMzAug	334	SEa + EMzAug
263	SnH + CMzSep	**275**	SnH + EaMzSep	**287**	SC + EaMzSep	**299**	SC + MMzSep	**311**	SA + EaMzSep	**323**	SEa + MMzSep	335	SEa + EMzSep
264	SnH + CMzOct	**276**	SnH + EaMzOct	**288**	SC + EaMzOct	**300**	SC + MMzOct	**312**	SA + EaMzOct	**324**	SEa + MMzOct	336	SEa + EMzOct
							Fruit						
TLC 29 (nH)		**TLC 30 (C)**		**TLC 31 (A)**		**TLC 32 (Ea)**		**TLC 33 (M)**		**TLC 34 (E)**		**TLC 35 (W)**	
337	FrnHIMay	**349**	FrCIMay	**361**	FrAIMay	**373**	FrEaIMay	**385**	FrMIMay	**397**	FrEIMay	409	FrWIMay
338	FrnHIJune	**350**	FrCIJune	**362**	FrAIJune	**374**	FrEaIJune	**386**	FrMIJune	**398**	FrEIJune	410	FrWIJune
339	FrnHIJuly	**351**	FrCIJuly	**363**	FrAIJuly	**375**	FrEaIJuly	**387**	FrMIJuly	**399**	FrEIJuly	411	FrWIJuly
340	FrnHIAug	**352**	FrCIAug	**364**	FrAIAug	**376**	FrEaIAug	**388**	FrMIAug	**400**	FrEIAug	412	FrWIAug
341	FrnHISep	**353**	FrCISep	**365**	FrAISep	**377**	FrEaISep	**389**	FrMISep	**401**	FrEISep	413	FrWISep
342	FrnHIOct	**354**	FrCIOct	**366**	FrAIOct	**378**	FrEaIOct	**390**	FrMIOct	**402**	FrEIOct	414	FrWIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
343	FrnHMzMay	**355**	FrCMzMay	**367**	FrAMzMay	**379**	FrEaMzMay	**391**	FrMMzMay	**403**	FrEMzMay	415	FrWMzMay
344	FrnHMzJune	**356**	FrCMzJune	**368**	FrAMzJune	**380**	FrEaMzJune	**392**	FrMzIJune	**404**	FrEMzJune	416	FrWMzJune
345	FrnHMzJuly	**357**	FrCMzJuly	**369**	FrAMzJuly	**381**	FrEaMzJuly	**393**	FrMzIJuly	**405**	FrEMzJuly	417	FrWMzJuly
346	FrnHMzAug	**358**	FrCMzAug	**370**	FrAMzAug	**382**	FrEaMzAug	**394**	FrMzIAug	**406**	FrEMzAug	418	FrWMzAug
347	FrnHMzSep	**359**	FrCMzSep	**371**	FrAMzSep	**383**	FREAMzSep	**395**	FrMzISep	**407**	FrEMzSep	419	FrWMzSep
348	FrnHMzOct	**360**	FrCMzOct	**372**	FrAMzOct	**384**	FrEaMzOct	**396**	FrMzIOct	**408**	FrEMzOct	420	FrWMzOct
TLC 36 (nH + C)		**TLC 37 (nH + Ea)**		**TLC 38 (C + Ea)**		**TLC 39 (C + M)**		**TLC 40 (A + Ea)**		**TLC 41 (Ea + M)**		**TLC 42 (Ea + E)**	
421	FrnH + CIMay	**433**	FrnH + EaIMay	**445**	FrC + EaIMay	**457**	FrC + MIMay	**469**	FrA + EaIMay	**481**	FrEa + MIMay	493	FrEa + EIMay
422	FrnH + CIJune	**434**	FrnH + EaIJune	**446**	FrC + EaIJune	**458**	FrC + MIJune	**470**	FrA + EaIJune	**482**	FrEa + MIJune	494	FrEa + EIJune
423	FrnH + CIJuly	**435**	FrnH + EaIJuly	**447**	FrC + EaIJuly	**459**	FrC + MIJuly	**471**	FrA + EaIJuly	**483**	FrEa + MIJuly	495	FrEa + EIJuly
424	FrnH + CIAug	**436**	FrnH + EaIAug	**448**	FrC + EaIAug	**460**	FrC + MIAug	**472**	FrA + EaIAug	**484**	FrEa + MIAug	496	FrEa + EIAug
425	FrnH + CISep	**437**	FrnH + EaISep	**449**	FrC + EaISep	**461**	FrC + MISep	**473**	FrA + EaISep	**485**	FrEa + MISep	497	FrEa + EISep
426	FrnH + CIOct	**438**	FrnH + EaIOct	**450**	FrC + EaIOct	**462**	FrC + MIOct	**474**	FrA + EaIOct	**486**	FrEa + MIOct	498	FrEa + EIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
427	FrnH + CMzMay	**439**	FrnH + EaMzMay	**451**	FrC + EaMzMay	**463**	FrC + MMzMay	**475**	FrA + EaMzMay	**487**	FrEa + MMzMay	499	FrEa + EMzMay
428	FrnH + CMzJune	**440**	FrnH + EaMzJune	**452**	FrC + EaMzJune	**464**	FrC + MMzJune	**476**	FrA + EaMzJune	**488**	FrEa + MMzJune	500	FrEa + EMzJune
429	FrnH + CMzJuly	**441**	FrnH + EaMzJuly	**453**	FrC + EaMzJuly	**465**	FrC + MMzJuly	**477**	FrA + EaMzJuly	**489**	FrEa + MMzJuly	501	FrEa + EMzJuly
430	FrnH + CMzAug	**442**	FrnH + EaMzAug	**454**	FrC + EaMzAug	**466**	FrC + MMzAug	**478**	FrA + EaMzAug	**490**	FrEa + MMzAug	502	FrEa + EMzAug
431	FrnH + CMzSep	**443**	FrnH + EaMzSep	**455**	FrC + EaMzSep	**467**	FrC + MMzSep	**479**	FrA + EaMzSep	**491**	FrEa + MMzSep	503	FrEa + EMzSep

(*continued on next page*)

Table 1 (*continued*)

						D. Innoxia							
						Leaves							
	TLC 1 (nH)		**TLC 2 (C)**		**TLC 3 (A)**		**TLC 4 (Ea)**		**TLC 5 (M)**		**TLC 6 (E)**		**TLC 7 (W)**
432	FrnH + CMzOct	**444**	FrnH + EaMzOct	**456**	FrC + EaMzOct	**468**	FrC + MMzOct	**480**	FrA + EaMzOct	**492**	FrEa + MMzOct	504	FrEa + EMzOct
						Flower							
	TLC 43 (nH)		**TLC 44 (C)**		**TLC 45 (A)**		**TLC 46 (Ea)**		**TLC 47 (M)**		**TLC 48 (E)**		**TLC 49 (W)**
505	FlnHIMay	**517**	FlCIMay	**529**	FlAIMay	**541**	FlEaIMay	**553**	FlMIMay	**565**	FlEIMay	577	FlWIMay
506	FlnHIJune	**518**	FlCIJune	**530**	FlAIJune	**542**	FlEaIJune	**554**	FlMIJune	**566**	FlEIJune	578	FlWIJune
507	FlnHIJuly	**519**	FlCIJuly	**531**	FlAIJuly	**543**	FlEaIJuly	**555**	FlMIJuly	**567**	FlEIJuly	579	FlWIJuly
508	FlnHIAug	**520**	FlCIAug	**532**	FlAIAug	**544**	FlEaIAug	**556**	FlMIAug	**568**	FlEIAug	580	FlWIAug
509	FlnHISep	**521**	FlCISep	**533**	FlAISep	**545**	FlEaISep	**557**	FlMISep	**569**	FlEISep	581	FlWISep
510	FlnHIOct	**522**	FlCIOct	**534**	FlAIOct	**546**	FlEaIOct	**558**	FlMIOct	**570**	FlEIOct	582	FlWIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
511	FlnHMzMay	**523**	FlCMzMay	**535**	FlAMzMay	**547**	FlEaMzMay	**559**	FlMMzMay	**571**	FlEMzMay	583	FlWMzMay
512	FlnHMzJune	**524**	FlCMzJune	**536**	FlAMzJune	**548**	FlEaMzJune	**560**	FlMzIJune	**572**	FlEMzJune	584	FlWMzJune
513	FlnHMzJuly	**525**	FlCMzJuly	**537**	FlAMzJuly	**549**	FlEaMzJuly	**561**	FlMzIJuly	**573**	FlEMzJuly	585	FlWMzJuly
514	FlnHMzAug	**526**	FlCMzAug	**538**	FlAMzAug	**550**	FlEaMzAug	**562**	FlMzIAug	**574**	FlEMzAug	586	FlWMzAug
515	FlnHMzSep	**527**	FlCMzSep	**539**	FlAMzSep	**551**	FREAMzSep	**563**	FlMzISep	**575**	FlEMzSep	587	FlWMzSep
516	FlnHMzOct	**528**	FlCMzOct	**540**	FlAMzOct	**552**	FlEaMzOct	**564**	FlMzIOct	**576**	FlEMzOct	588	FlWMzOct
	TLC 50 (nH + C)		**TLC 51 (nH + Ea)**		**TLC 52 (C + Ea)**		**TLC 53 (C + M)**		**TLC 54 (A + Ea)**		**TLC 55 (Ea + M)**		**TLC 56 (Ea + E)**
589	FlnH + CIMay	**601**	FlnH + EaIMay	**613**	FlC + EaIMay	**625**	FlC + MIMay	**637**	FlA + EaIMay	**649**	FlEa + MIMay	661	FlEa + EIMay
590	FlnH + CIJune	**602**	FlnH + EaIJune	**614**	FlC + EaIJune	**626**	FlC + MIJune	**638**	FlA + EaIJune	**650**	FlEa + MIJune	662	FlEa + EIJune
591	FlnH + CIJuly	**603**	FlnH + EaIJuly	**615**	FlC + EaIJuly	**627**	FlC + MIJuly	**639**	FlA + EaIJuly	**651**	FlEa + MIJuly	663	FlEa + EIJuly
592	FlnH + CIAug	**604**	FlnH + EaIAug	**616**	FlC + EaIAug	**628**	FlC + MIAug	**640**	FlA + EaIAug	**652**	FlEa + MIAug	664	FlEa + EIAug
593	FlnH + CISep	**605**	FlnH + EaISep	**617**	FlC + EaISep	**629**	FlC + MISep	**641**	FlA + EaISep	**653**	FlEa + MISep	665	FlEa + EISep
594	FlnH + CIOct	**606**	FlnH + EaIOct	**618**	FlC + EaIOct	**630**	FlC + MIOct	**642**	FlA + EaIOct	**654**	FlEa + MIOct	666	FlEa + EIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
595	FlnH + CMzMay	**607**	FlnH + EaMzMay	**619**	FlC + EaMzMay	**631**	FlC + MMzMay	**643**	FlA + EaMzMay	**655**	FlEa + MMzMay	667	FlEa + EMzMay
596	FlnH + CMzJune	**608**	FlnH + EaMzJune	**620**	FlC + EaMzJune	**632**	FlC + MMzJune	**644**	FlA + EaMzJune	**656**	FlEa + MMzJune	668	FlEa + EMzJune
597	FlnH + CMzJuly	**609**	FlnH + EaMzJuly	**621**	FlC + EaMzJuly	**633**	FlC + MMzJuly	**645**	FlA + EaMzJuly	**657**	FlEa + MMzJuly	669	FlEa + EMzJuly
598	FlnH + CMzAug	**610**	FlnH + EaMzAug	**622**	FlC + EaMzAug	**634**	FlC + MMzAug	**646**	FlA + EaMzAug	**658**	FlEa + MMzAug	670	FlEa + EMzAug
599	FlnH + CMzSep	**611**	FlnH + EaMzSep	**623**	FlC + EaMzSep	**635**	FlC + MMzSep	**647**	FlA + EaMzSep	**659**	FlEa + MMzSep	671	FlEa + EMzSep
600	FlnH + CMzOct	**612**	FlnH + EaMzOct	**624**	FlC + EaMzOct	**636**	FlC + MMzOct	**648**	FlA + EaMzOct	**660**	FlEa + MMzOct	672	FlEa + EMzOct
						Root							
	TLC 57 (nH)		**TLC 58 (C)**		**TLC 59 (A)**		**TLC60 (Ea)**		**TLC 61 (M)**		**TLC 62 (E)**		**TLC 63 (W)**
673	RnHIMay	**685**	RCIMay	**697**	RAIMay	**709**	REaIMay	**721**	RMIMay	**733**	REIMay	745	RWIMay
674	RnHIJune	**686**	RCIJune	**698**	RAIJune	**710**	REaIJune	**722**	RMIJune	**734**	REIJune	746	RWIJune
675	RnHIJuly	**687**	RCIJuly	**699**	RAIJuly	**711**	REaIJuly	**723**	RMIJuly	**735**	REIJuly	747	RWIJuly
676	RnHIAug	**688**	RCIAug	**700**	RAIAug	**712**	REaIAug	**724**	RMIAug	**736**	REIAug	748	RWIAug
677	RnHISep	**689**	RCISep	**701**	RAISep	**713**	REaISep	**725**	RMISep	**737**	REISep	749	RWISep
678	RnHIOct	**690**	RCIOct	**702**	RAIOct	**714**	REaIOct	**726**	RMIOct	**738**	REIOct	750	RWIOct
	Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**
679	RnHMzMay	**691**	RCMzMay	**703**	RAMzMay	**715**	REaMzMay	**727**	RMMzMay	**739**	REMzMay	751	RWMzMay
680	RnHMzJune	**692**	RCMzJune	**704**	RAMzJune	**716**	REaMzJune	**728**	RMzIJune	**740**	REMzJune	752	RWMzJune
681	RnHMzJuly	**693**	RCMzJuly	**705**	RAMzJuly	**717**	REaMzJuly	**729**	RMzIJuly	**741**	REMzJuly	753	RWMzJuly

Table 1 (*continued*)

D. Innoxia													
Leaves													
TLC 1 (nH)		**TLC 2 (C)**		**TLC 3 (A)**		**TLC 4 (Ea)**		**TLC 5 (M)**		**TLC 6 (E)**		**TLC 7 (W)**	
682	RnHMzAug	**694**	RCMzAug	**706**	RAMzAug	**718**	REaMzAug	**730**	RMzIAug	**742**	REMzAug	754	RWMzAug
683	RnHMzSep	**695**	RCMzSep	**707**	RAMzSep	**719**	FREAMzSep	**731**	RMzISep	**743**	REMzSep	755	RWMzSep
684	RnHMzOct	**696**	RCMzOct	**708**	RAMzOct	**720**	REaMzOct	**732**	RMzIOct	**744**	REMzOct	756	RWMzOct
TLC 64 (nH + C)		**TLC 65 (nH + Ea)**		**TLC 66 (C + Ea)**		**TLC 67 (C + M)**		**TLC 68 (A + Ea)**		**TLC 69 (Ea + M)**		**TLC 70 (Ea + E)**	
757	RnH + CIMay	**769**	RnH + EaIMay	**781**	RC + EaIMay	**793**	RC + MIMay	**805**	RA + EaIMay	**817**	REa + MIMay	**829**	REa + EIMay
758	RnH + CIJune	**770**	RnH + EaIJune	**782**	RC + EaIJune	**794**	RC + MIJune	**806**	RA + EaIJune	**818**	REa + MIJune	**830**	REa + EIJune
759	RnH + CIJuly	**771**	RnH + EaIJuly	**783**	RC + EaIJuly	**795**	RC + MIJuly	**807**	RA + EaIJuly	**819**	REa + MIJuly	**831**	REa + EIJuly
760	RnH + CIAug	**772**	RnH + EaIAug	**784**	RC + EaIAug	**796**	RC + MIAug	**808**	RA + EaIAug	**820**	REa + MIAug	**832**	REa + EIAug
761	RnH + CISep	**773**	RnH + EaISep	**785**	RC + EaISep	**797**	RC + MISep	**809**	RA + EaISep	**821**	REa + MISep	**833**	REa + EISep
762	RnH + CIOct	**774**	RnH + EaIOct	**786**	RC + EaIOct	**798**	RC + MIOct	**810**	RA + EaIOct	**822**	REa + MIOct	**834**	REa + EIOct
Standard		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**		**Standard**	
763	RnH + CMzMay	**775**	RnH + EaMzMay	**787**	RC + EaMzMay	**799**	RC + MMzMay	**811**	RA + EaMzMay	**823**	REa + MMzMay	**835**	REa + EMzMay
764	RnH + CMzJune	**776**	RnH + EaMzJune	**788**	RC + EaMzJune	**800**	RC + MMzJune	**812**	RA + EaMzJune	**824**	REa + MMzJune	**836**	REa + EMzJune
765	RnH + CMzJuly	**777**	RnH + EaMzJuly	**789**	RC + EaMzJuly	**801**	RC + MMzJuly	**813**	RA + EaMzJuly	**825**	REa + MMzJuly	**837**	REa + EMzJuly
766	RnH + CMzAug	**778**	RnH + EaMzAug	**790**	RC + EaMzAug	**802**	RC + MMzAug	**814**	RA + EaMzAug	**826**	REa + MMzAug	**838**	REa + EMzAug
767	RnH + CMzSep	**779**	RnH + EaMzSep	**791**	RC + EaMzSep	**803**	RC + MMzSep	**815**	RA + EaMzSep	**827**	REa + MMzSep	**839**	REa + EMzSep
768	RnH + CMzOct	**780**	RnH + EaMzOct	**792**	RC + EaMzOct	**804**	RC + MMzOct	**816**	RA + EaMzOct	**828**	REa + MMzOct	**840**	REa + EMzOct

Normal phase thin layer chromatography (TLC), Leaves (L), stem (S), fruit (Fr), flower (Fl) root (R) Islamabad (I), Muzaffargarh (Mz), n-hexane (nH), chloroform (C), acetone (A), ethyl acetate (Ea), methanol (M), ethanol (E), water (W), August (Aug), September (Sep) and October (Oct). Standard = withametelin and daturaolone. TLC optimization of standards was finalized. Samples were analyzed on 4 * 6.66 cm TLC plates. 1 µl of each plant sample was spotted on TLC plate and elution was done. Each TLC analysis was performed in triplicate.

Table 2 RP HPLC method optimization parameters (linearity, sensitivity) values of withametelin and daturaolone.

Compound	Linearity (μg/ml)	Retention Time (Min)	Correlation coefficent	LOD (μg)	LOQ (μg)
Withametelin	10–0.31	12.0	0.99	0.1	0.5
Daturaolone	10–0.31	14.2	0.99	0.2	0.7

Table 3 Accuracy determination by analyzing withametelin and daturaolone at selected concentrations.

Analyte/Initial Concentration	Theoretical concentration after dilution added in the sample (μg/mL)	Amount recovered (μg/mL)	Recovery (%)	Mean (%)	RSD (%)
Withametelin (Concentration measured in the sample (LEa + EMzMay) = 3.96 μg)	0.5	4.48	100.65	100.74	0.33
		4.51	101.20		
		4.47	100.38		
	1.0	4.94	99.62	100.41	0.72
		4.97	100.25		
		5.02	101.38		
	1.5	5.45	99.94	100.62	0.65
		5.48	100.42		
		5.54	101.51		
Daturaolone (Concentration measured in the sample (FrEa + EIJuly) = 4.55 μg)	0.7	5.21	99.25	99.68	0.83
		5.29	100.84		
		5.19	98.95		
	1.4	6.02	101.25	100.52	0.51
		5.95	100.08		
		5.96	100.24		
	2.1	6.64	99.90	100.59	0.60
		6.74	101.38		
		6.68	100.51		

Table 4 Results of precision tests at selected concentrations.

Analyte	Concentration	Intra-day precision (n = 3)		Inter-day precision (n = 3)	
		RSD %	accuracy	RSD%	Accuracy
Withametelin	1.25	0.36	100.56	0.32	100.61
	2.5	0.45	99.87	0.49	100.24
	5	0.48	100.28	0.52	99.95
Daturaolone	1.25	0.78	99.58	0.80	100.59
	2.5	0.52	101.20	0.61	101.92
	5	0.59	100.73	0.44	100.26

telin content in *D. innoxia* was found to be correlated ($P < 0.05$) with temperature. High temperatures result in heat stress which affect plant secondary metabolites production. Cold stress also has a negative impact on plant growth and development, resulting in significant productivity constraints. It prevents plants from expressing their full genetic potential, directly inhibiting metabolic reactions, indirectly preventing water absorption and cell dehydration (Verma and Shukla, 2015). Our study showed that heat and cold stress had an impact on the variations in withametelin and daturaolone content. Humidity parameters were relatively high in the July, August and September in Islamabad region as compared to Muzaffargarh region. High humidity can exacerbate the harmful effects of high temperature by limiting transpiration. (i.e., moisture loss from leaves). This is essential to reduce leaf surface temperature and promote the absorption and mobility of water and minerals. Furthermore, high humidity increases the harmful effects of air pollution (such as ozone) and promotes infection spreading by increasing the size of the stomatal openings (Yang et al., 2012). Daturaolone content in *D. innoxia* was found to be correlated ($P < 0.01$) with humidity where its presence was found to be highest in August in I where the humidity value was also highest. Similarly, surface soil wetness in Multan was below 0.2 and root zone soil wetness was below 0.3 in six month period measurements. In a drought-stricken situation, the water available in the soil falls to critical levels, and atmospheric conditions increase the continuing loss of water. The severity of the water shortage is thought to reduce plant growth, but some studies have shown that water stress can increase secondary metabolites (Yang et al., 2012). Daturaolone content varied with soil wetness and quantified values showed significant ($P < 0.01$) value. Six month intra-variations in Islamabad were also observed for UVA irradiation. But no correlation was found between extent of UVA radiations and the quantified content of withametelin and daturaolone. The use of a controlled environment research to

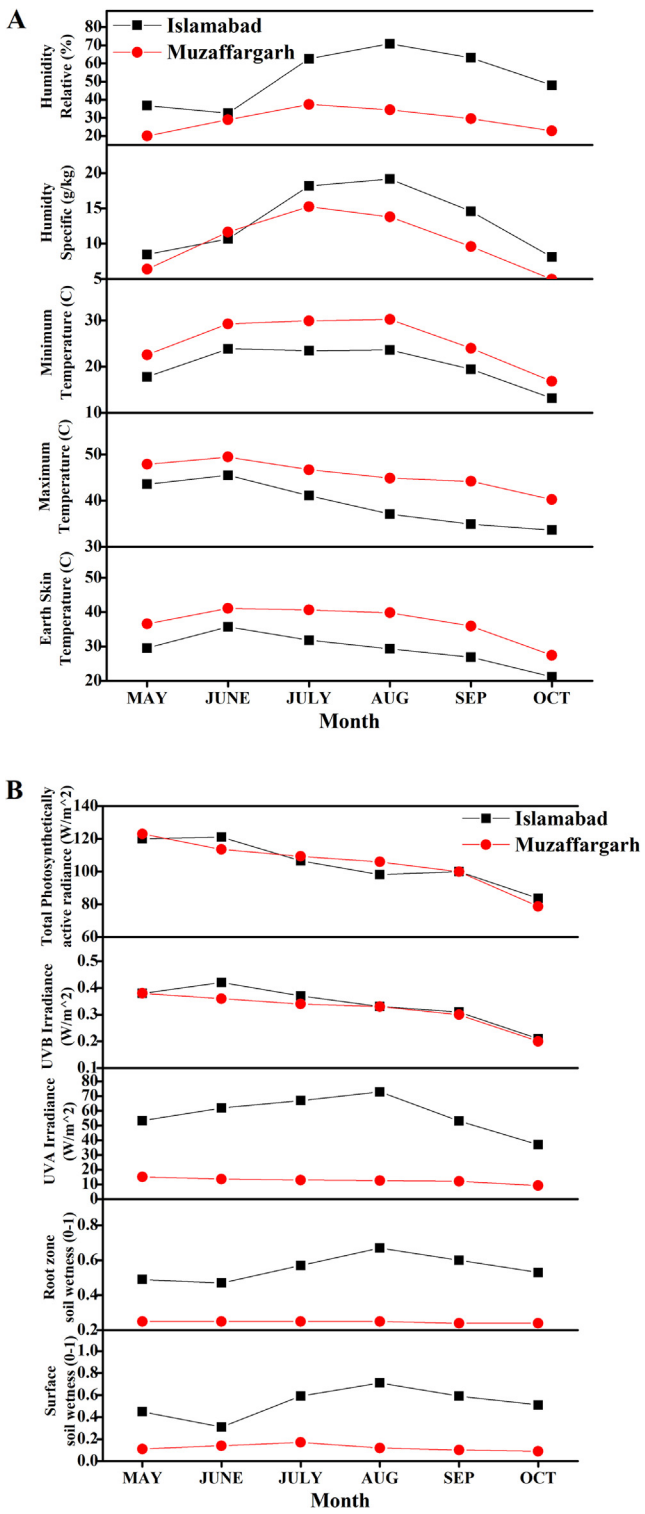

Fig. 1 Agroclimatology data (A = temperature and humidity parameters while B = drought stress, UV irradiance) was obtained for the study. The detailed agroclimatology reports of 1 year (January 2018 to December 2018) of selected sites were downloaded in CSV format and 6-month agroclimatology data was utilized in the current project. The data was obtained from the National Aeronautics and Space Administration (NASA) Langley Research Center (LaRC) Prediction of Worldwide Energy Resource (POWER) Project funded through the NASA Earth Science/Applied Science Program.

determine the quantitative relationship between various parameters with more accuracy is proposed.

3.2. TLC method optimization showed Nh:EA (70:30) for co detection of withametelin and daturaolone

The robustness and sustainability of planar chromatography techniques play an important role in the quality assessment of pharmaceutical products in resource-limited countries (Kaale et al., 2011). Advantages of TLC methods that other techniques will never achieve include its simplicity, high throughput and simultaneous analysis of multiple complex samples (Ferenczi-Fodor et al., 2006) So, for the development of appropriate bands to detect withametelin and daturaolone, normal phase TLC technique was utilized. Various combined ratios (v/v) of n-hexane (nH) and ethyl acetate (Ea) were checked. It includes: nH: Ea (1:1), nH: Ea (3:2), nH: Ea (3.5:1.5), nH: Ea (4:1), nH: Ea (8.5:1.5), and nH: Ea (4.5:0.5). The mobile phase combinations i.e. nH: Ea (1:1), nHa: Ea (3:2), nH: Ea (4:1), nH: Ea (8.5:1.5) and nH: Ea (4.5:0.5) revealed unsatisfactory chromatographic separations and detection of the compounds. When mobile phase nH: Ea (70:30) was evaluated, it provided well-resolved and intact chromatographic detections for withametelin and daturaolone. Consequently, the nH:Ea (70:30) was selected for the co-detection of withametelin and daturaolone in all prepared samples for the TLC analysis.

3.2.1. TLC analysis showed the detections in 118/840 samples

TLC analysis of all 840 samples (Table) with standards were run using the mobile phase optimized for the co-detection of withametelin and daturaolone (Fig. 2). Detection of withametelin was mostly observed in leaf samples, especially in TLC 4, 13 and 14 (Fig. 2A) where ethyl acetate, ethyl acetate-methanol (1:1) and ethyl acetate-ethanol (1:1) are the extraction medium. All samples which show detection of withametelin in different samples of leaves are given in Table 3. None of the samples from the root, fruit, flower and stem portion showed the detection of withametelin. Whereas detection of daturaolone was observed in fruit samples, especially in TLC 34 and 42 where ethyl acetate and ethyl acetate-ethanol (1:1) are the extraction medium (Fig. 2B). None of the samples from root, leaves, flower and stem portion showed the detection of daturaolone. The visualizing effect depends on the chemical structure of the detecting reagent, detected substance, and the chromatographic adsorbent used. In particular, the application of visualization reagent reacts with the substances present in the analyzed mixture and gives diversified colors of chromatographic spots (Pyka, 2014).

3.3. RP HPLC method was developed

High-performance liquid chromatography (HPLC) is a modern, powerful, and flexible separation technology that is usually used to separate, identify and quantify components of herbal mixtures to obtain their chemical profiles (Sarker and Nahar, 2015). The parameters for analysis of withametelin and daturaolone were determined for the first time by adjusting their analytical parameters respectively. It is aimed at identifying the best conditions for the analysis of compounds. Optimization was carried out using gradient elution for each

Table 5 RP HPLC quantification analysis of withametelin and daturaolone in selected samples of *D. innoxia*.

Withametelin (μg/mg dry powder)														
13	**LCIMay**	**1.19 ± 0.21**	**40**	**LEaIAug**	**1.31 ± 0.14**	**68**	**LEMzJune**	**3.85 ± 0.21**	**128**	**LC + MMzJune**	**3.65 ± 0.38**	**156**	**LEa + MMzOct**	**0.61 ± 0.02**
14	**LCIJune**	**1.43 ± 0.33**	**43**	**LEaMzMay**	**2.68 ± 0.42**	**69**	**LEMzJuly**	**3.32 ± 0.34**	**139**	**LA + EaMzMay**	**3.29 ± 0.41**	**157**	**LEa + EIMay**	**1.28 ± 0.03**
17	**LCISep**	**0.77 ± 0.24**	**44**	**LEaMzJune**	**2.94 ± 0.13**	**70**	**LEMzAug**	**1.19 ± 0.18**	**140**	**LA + EaMzJune**	**3.55 ± 0.42**	**158**	**LEa + EIJune**	**2.15 ± 0.21**
18	**LCIOct**	**0.58 ± 0.14**	**49**	**LMIMay**	**2.19 ± 0.13**	**71**	**LEMzSep**	**0.93 ± 0.31**	**145**	**LEa + MIMay**	**0.72 ± 0.02**	**159**	**LEa + EIJuly**	**0.96 ± 0.19**
20	**LCMzJune**	**1.47 ± 0.12**	**50**	**LMIJune**	**3.52 ± 0.21**	**92**	**LnH + CMzJune**	**0.94 ± 0.09**	**146**	**LEa + MIJune**	**1.28 ± 0.03**	**161**	**LEa + EISep**	**0.64 ± 0.11**
23	**LCMzSep**	**0.63 ± 0.13**	**54**	**LMIOct**	**0.63 ± 0.32**	**97**	**LnH + EaIMay**	**1.19 ± 0.04**	**147**	**LEa + MIJuly**	**0.65 ± 0.02**	**162**	**LEa + EIOct**	**0.68 ± 0.05**
24	**LCMzOct**	**0.55 ± 0.09**	**55**	**LMMzMay**	**3.81 ± 0.41**	**98**	**LnH + EaIJune**	**2.06 ± 0.23**	**149**	**LEa + MISep**	**0.41 ± 0.01**	**163**	**LEa + EMzMay**	**3.96 ± 0.32**
25	**LAIMay**	**1.35 ± 0.16**	**56**	**LMMzJune**	**4.48 ± 0.25**	**103**	**LnH + EaMzMay**	**1.58 ± 0.41**	**150**	**LEa + MIOct**	**0.39 ± 0.02**	**164**	**LEa + EMzJune**	**5.12 ± 0.28**
26	**LAIJune**	**1.82 ± 0.20**	**61**	**LEIMay**	**3.73 ± 0.31**	**104**	**LnH + EaMzJune**	**2.24 ± 0.51**	**151**	**LEa + MMzMay**	**1.34 ± 0.04**	**165**	**LEa + EMzJuly**	**4.66 ± 0.22**
27	**LAIJuly**	**1.61 ± 0.33**	**62**	**LEIJune**	**3.81 ± 0.33**	**107**	**LnH + EaMzSep**	**0.92 ± 0.32**	**152**	**LEa + MMzJune**	**3.15 ± 0.02**	**167**	**LEa + EMzAug**	**1.24 ± 0.37**
32	**LAMzJune**	**2.19 ± 0.37**	**63**	**LEIJuly**	**2.69 ± 0.23**	**108**	**LnH + EaMzOct**	**0.78 ± 0.33**	**153**	**LEa + MMzJuly**	**1.09 ± 0.03**	**168**	**LEa + EMzSep**	**0.76 ± 0.05**
38	**LEaIJune**	**1.93 ± 0.21**	**67**	**LEMzMay**	**2.47 ± 0.12**	**116**	**LC + EaMzJune**	**2.68 ± 0.56**	**155**	**LEa + MMzSep**	**0.74 ± 0.04**			
Daturaolone (μg/mg dry powder)														
349	**FrCIMay**	**2.19 ± 0.39**	**378**	**FrEaIOct**	**1.14 ± 0.24**	**437**	**FrnH + EaISep**	**0.91 ± 0.23**	**470**	**FrA + EaIJune**	**1.34 ± 0.26**	**488**	**FrEa + MMzJune**	**3.67 ± 0.32**
350	**FrCIJune**	**3.21 ± 0.21**	**388**	**FrMIAug**	**2.92 ± 0.21**	**438**	**FrnH + EaIOct**	**0.84 ± 0.11**	**471**	**FrA + EaIJuly**	**1.31 ± 0.17**	**489**	**FrEa + MMzJuly**	**3.49 ± 0.47**
351	**FrCIJuly**	**1.89 ± 0.24**	**389**	**FrMISep**	**2.63 ± 0.32**	**446**	**FrC + EaIJune**	**0.93 ± 0.16**	**472**	**FrA + EaIAug**	**1.15 ± 0.17**	**492**	**FrEa + MMzOct**	**2.66 ± 0.29**
352	**FrCIAug**	**0.93 ± 0.13**	**390**	**FrMIOct**	**2.11 ± 0.15**	**447**	**FrC + EaIJuly**	**0.84 ± 0.14**	**473**	**FrA + EaISep**	**0.85 ± 0.44**	**495**	**FrEa + EIJune**	**4.21 ± 0.43**
353	**FrCISep**	**0.88 ± 0.14**	**398**	**FrEIJune**	**4.33 ± 0.24**	**449**	**FrC + EaISep**	**0.97 ± 0.09**	**474**	**FrA + EaIOct**	**0.82 ± 0.07**	**496**	**FrEa + EIJuly**	**4.55 ± 0.40**
354	**FrCIOct**	**0.93 ± 0.16**	**399**	**FrEIJuly**	**2.66 ± 0.44**	**450**	**FrC + EaIOct**	**0.93 ± 0.08**	**476**	**FrA + EaMzJune**	**1.22 ± 0.07**	**497**	**FrEa + EIAug**	**5.18 ± 0.45**
360	**FrCMzOct**	**0.82 ± 0.21**	**400**	**FrEIAug**	**2.38 ± 0.53**	**459**	**FrC + MIJuly**	**1.65 ± 0.20**	**477**	**FrA + EaMzJuly**	**0.86 ± 0.08**	**498**	**FrEa + EISep**	**4.76 ± 0.42**
373	**FrEaIMay**	**2.36 ± 0.33**	**401**	**FrEISep**	**1.62 ± 0.33**	**460**	**FrC + MIAug**	**1.30 ± 0.07**	**483**	**FrEa + MIJune**	**2.87 ± 0.21**	**500**	**FrEa + EMzJune**	**2.44 ± 0.38**
374	**FrEaIJune**	**3.44 ± 0.25**	**402**	**FrEIOct**	**1.52 ± 0.12**	**461**	**FrC + MISep**	**0.99 ± 0.09**	**484**	**FrEa + MIJuly**	**2.38 ± 0.28**	**501**	**FrEa + EMzJuly**	**2.31 ± 0.40**
375	**FrEaIJuly**	**3.31 ± 0.27**	**407**	**FrEMzSep**	**0.86 ± 0.19**	**462**	**FrC + MIOct**	**0.86 ± 0.11**	**485**	**FrEa + MIAug**	**2.03 ± 0.31**	**503**	**FrEa + EMzSep**	**2.02 ± 0.31**
376	**FrEaIAug**	**3.18 ± 0.33**	**408**	**FrEMzOct**	**0.89 ± 0.12**	**467**	**FrC + MMzSep**	**0.94 ± 0.05**	**486**	**FrEa + MISep**	**1.68 ± 0.33**	**504**	**FrEa + EMzOct**	**2.42 ± 0.37**
377	**FrEaISep**	**2.64 ± 0.35**	**436**	**FrnH + EaIAug**	**0.92 ± 0.11**	**468**	**FrC + MMzOct**	**0.58 ± 0.10**	**487**	**FrEa + MMzMay**	**3.24 ± 0.31**			

Normal phase thin layer chromatography (TLC), Leaves (L), fruit (Fr), Islamabad (I), Muzaffargarh (Mz), n-hexane (nH), chloroform (C), acetone (A), ethyl acetate (Ea), methanol (M), ethanol (E), water (W), August (Aug), September (Sep) and October (Oct). 118 samples that gave positive detections in TLC analysis were further analyzed for quantification analysis via RP HPLC. RP HPLC results are shown as mean ± standard deviation after triplicate analysis.

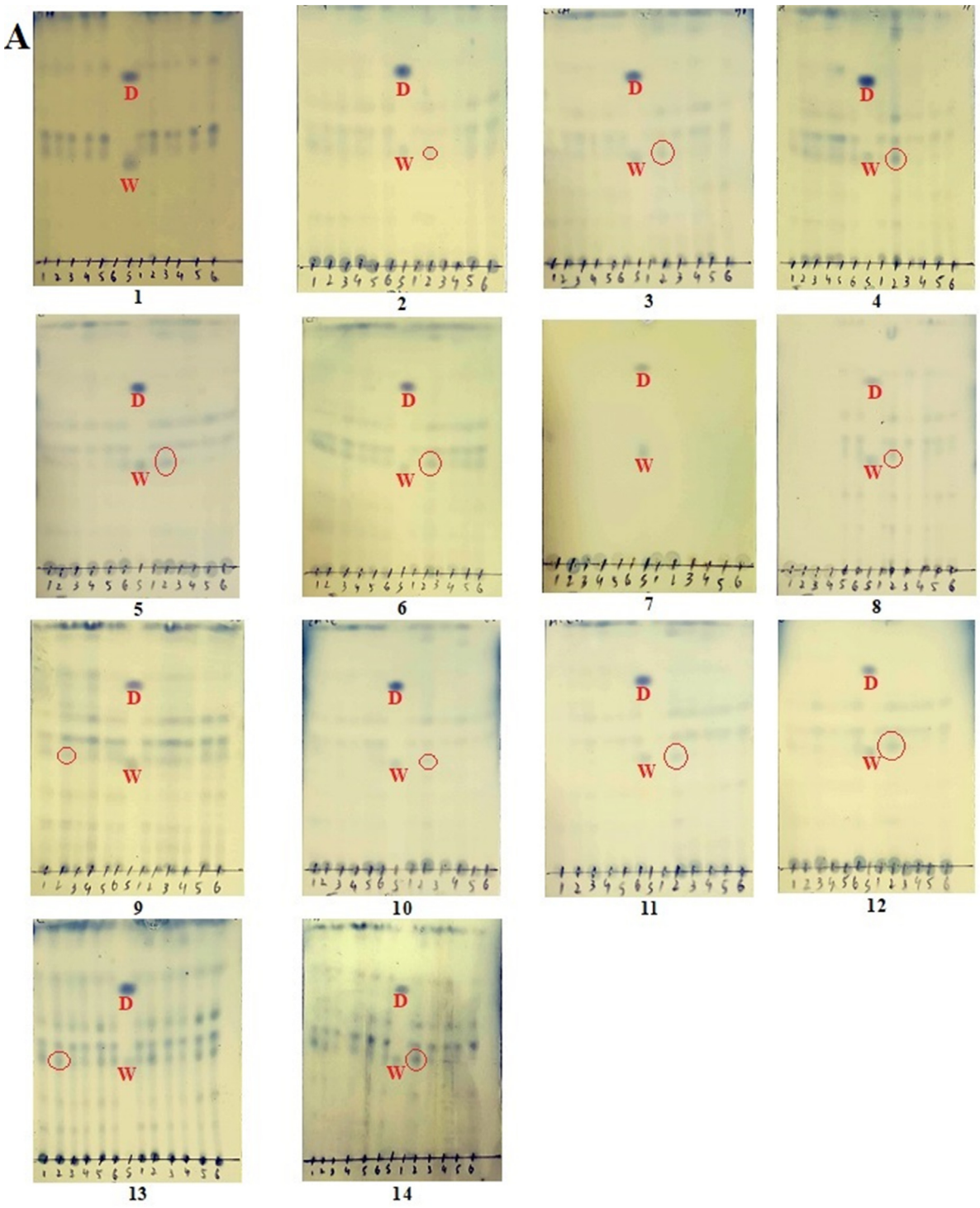

Fig. 2 TLC detection (red circled) of withametelin (W) and daturaolone (D) in selected samples of *D. innoxia* leaves (A) and fruits (B). TLC method was optimized for the co-detection of withametelin and daturaolone. 1 µl of the standard solution was run in different mobile phases to select the best mobile phase for separation, elution and simultaneous detection of withametelin and daturaolone. Phosphomolybdic acid reagent was used for the final detection and analysis.

compound. Subsequently, the time and composition of the eluent were adjusted until the optimal conditions were achieved. Moreover, gradient time changes are also used as an optimized parameter. Standard solutions of withametelin and daturaolone were injected. Data is processed using software linked to the HPLC system. Chromatogram met the criteria necessary to identify withametelin and daturaolone. In the absence of a valid method, a new method for analyzing new products is

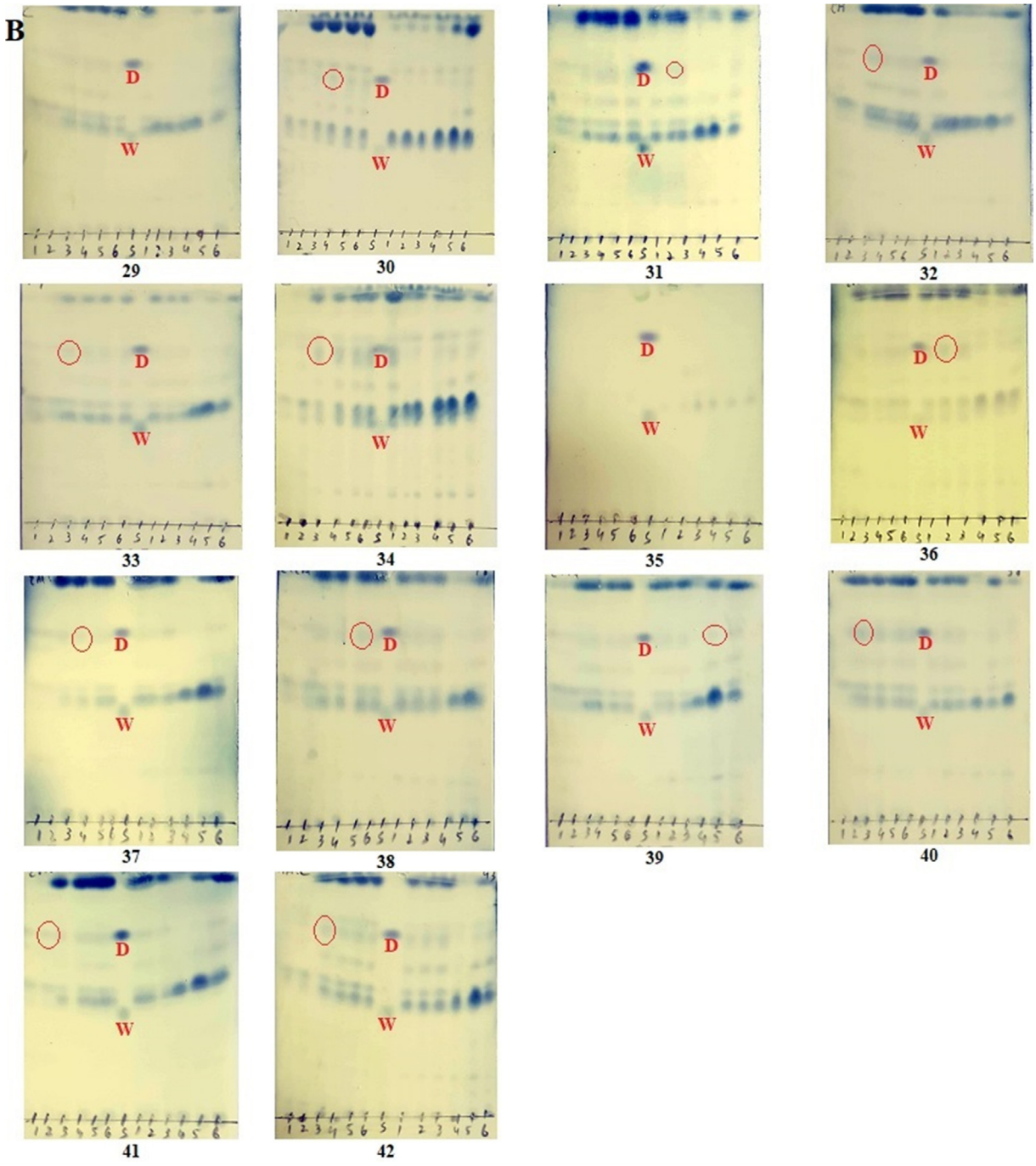

Fig. 2 (*continued*)

being developed. These methods are optimized and verified by test runs. An alternative method is proposed to replace the existing methodology in comparison laboratory data and implement it in practice, including all available benefits and disadvantages (Patil, 2017).

3.3.1. Optimization of chromatographic conditions

The first test was a single injection of standard withametelin and daturaolone at 500 ppm, injection volume being 50 µl. The various composition of mobile phase systems (methanol–water and methanol (100%)) was studied to obtain good chromatographic properties. Consequently, methanol-water (1:1) to 100% methanol was selected as a gradient system with the best elution behaviour. The limitation of the gradient elution system is the formation of ghost peaks, as shown by the standard daturaolone chromatogram at 254 nm (Fig. 2B). HPLC's "ghost peak" can be caused by dilution of samples, contamination of reagents and inorganic impurities such as nitrates, organic substances in dissolved plastic containers and synthetic impurities such as methanol and acetonitrile.

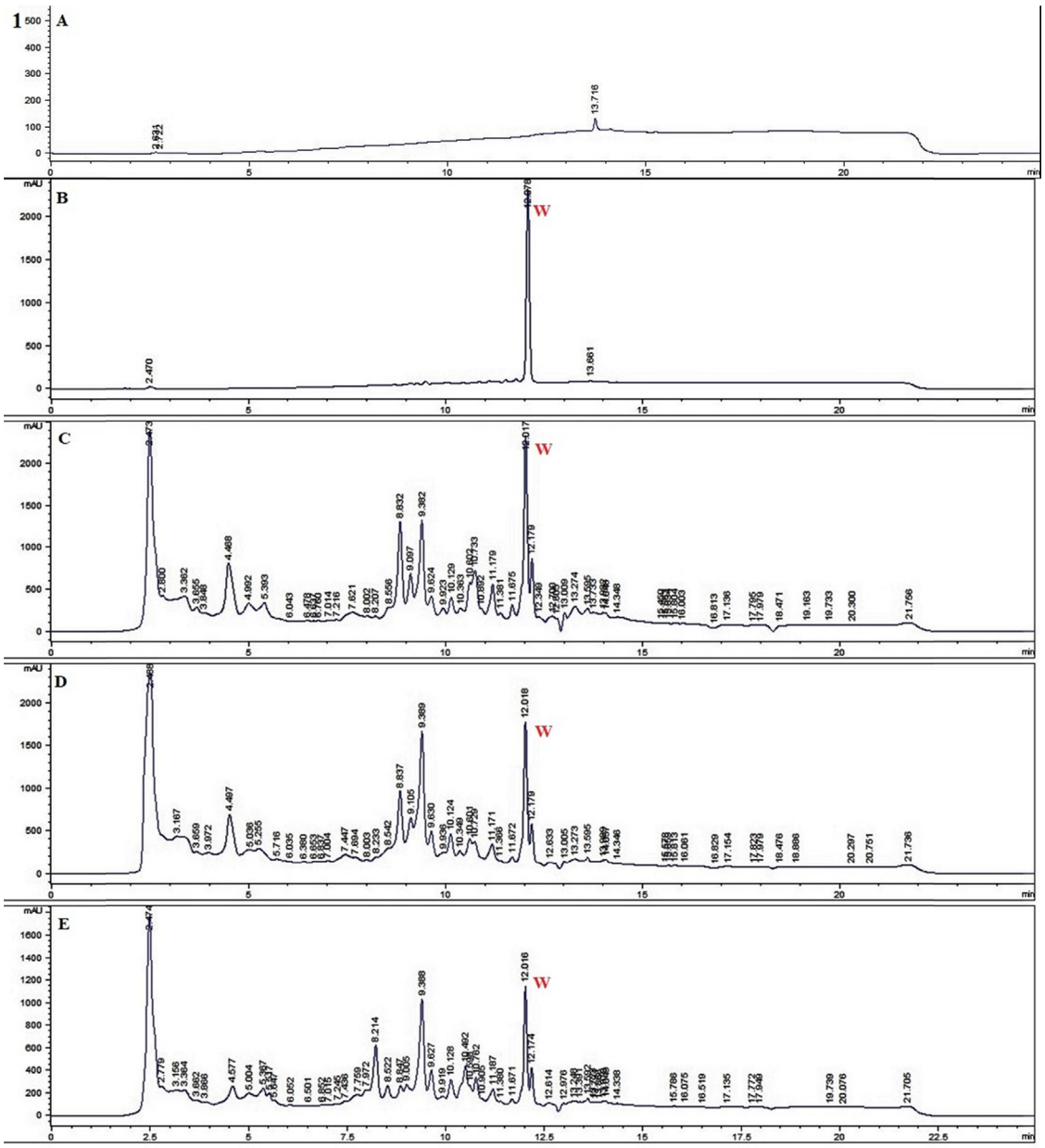

Fig. 3 RP HPLC chromatograms of selected samples for detection (Red colour) of withametelin (1) and daturaolone (2). 1 = withametelin (W) blank (A), standard withametelin (B) LEa + EMzJune (C) LEMzJune (D) and LC + EaMzJune (E). 2 = daturaolone (D) blank (A) standard daturaolone (B) FrEa + EIJune (C), FrEa + MIJune (D) and FrA + EaIJune (E) of *Datura innoxia*.

Even surfaces of glass containing detergent residues may cause an issue (SULASTRI et al., 2020). However, they did not affect the elution and quantification analysis.

3.3.2. Optimization of sample preparation conditions

Ultra sound assisted solid-liquid extracts from dry powders were obtained for the preparation of samples. Initially, the sample was dissolved using 1 mL of the first mobile phase. Results showed that this method was not satisfactory in terms of solubility and detection of the two compounds. However, methanol as a solubility agent produced good results. In combination with HPLC and suitable detectors, appropriate sample preparation techniques can provide valuable data for targeted applications. Proper sample preparation for HPLC results in efficient extraction, cleanup, and preconcentration in a single step, thus providing a pathway to tackle complex extract loading on HPLC. Ultrasonic assisted extraction is a state-of-the-art sampling technique that uses ultrasound waves

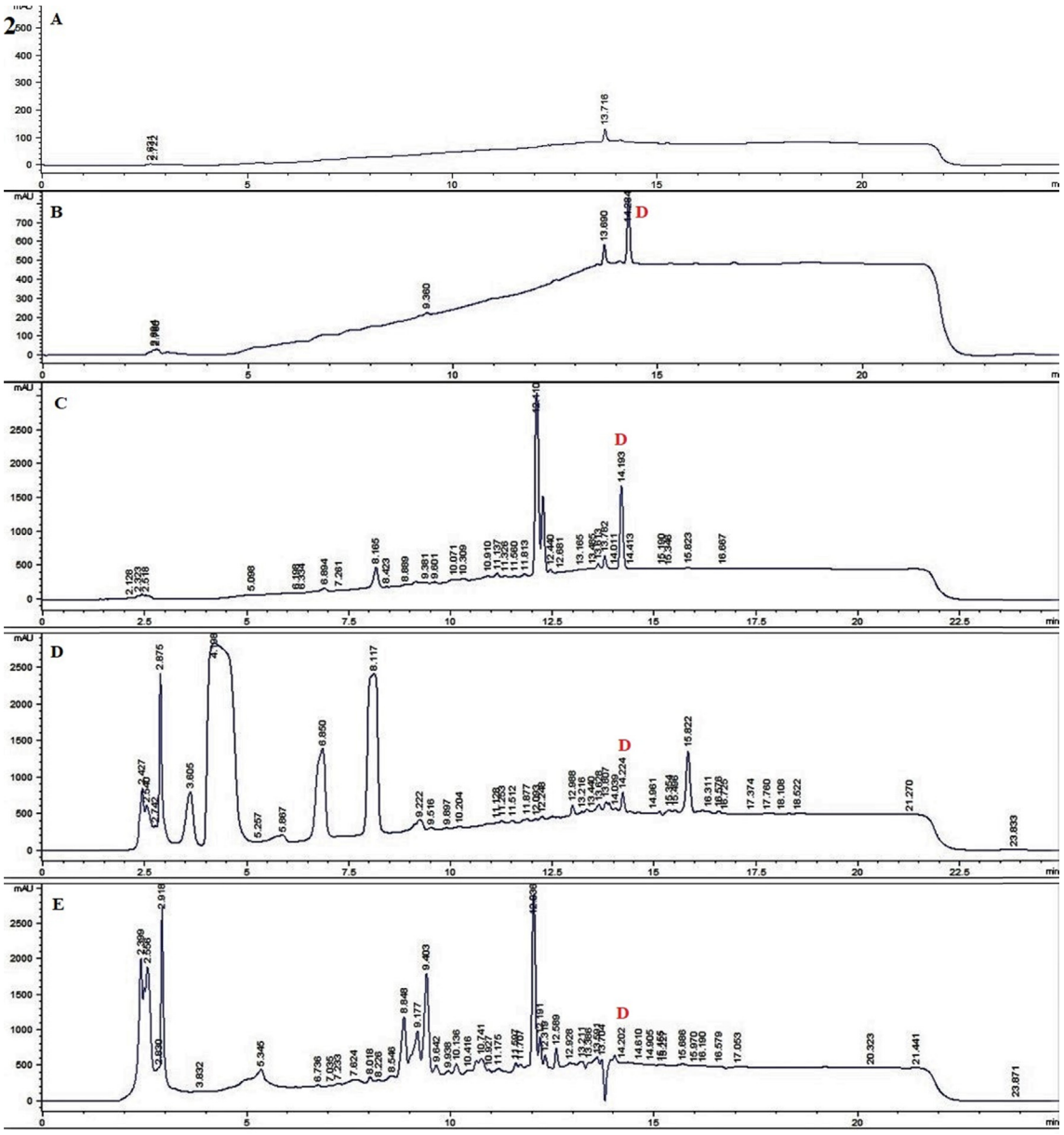

Fig. 3 (*continued*)

to extract many compounds from a complex matrix. It provides higher extraction output and faster kinetics than other conventional extraction methods (Kanu, 2021).

3.3.3. Linearity, LOD and LOQ

The excellent relationship between the linearity and the standard analysis is shown in Table 2, with "Y" being the peak area ratio and "X" being the concentration of the analysis contained in the extracted sample, respectively. Calibration curves of withametelin and daturaolone were determined for five concentrations in the range of 0.31–10 ppm, respectively. LOD and LOQ values are also shown in Table 2.

3.3.4. Accuracy and precision

The retrieval of compounds was determined using a spiked sample with a known amount of withametelin and daturaolone standards. The recovered amounts were calculated from the found total and the original amount. The results are shown in Table 3, in line with the recommendations of the ICT (Guideline, 2005). The intra and inter-day precision data are shown in Table 4. The results show that the variation coefficient is lower than the recommended value i.e. 5%. There were no significant differences in the results of the intra-day and inter-day tests, indicating that the accuracy of the proposed method was satisfactory.

3.3.5. Robustness

The robustness of HPLC method had been evaluated to ensure that it was not sensitive to small changes under experimental conditions. In this study, the wavelength, column supplier and pH of the mobile phase were changed. None of these changes led to a significantly different responses in peaks of withametelin and daturaolone.

Table 6 Correlation of quantified values in best extraction solvent (Ea + E) of withametelin and daturaolone with agroclimatic parameters.

Analyte	Agroclimatic Parameter	Correlation R^2	P value
Withametelin	Temperature	0.8	< 0.05
	Humidity	–	–
	UVA index	–	–
	Soil Wetness	–	–
Daturaolone	Temperature	–	–
	Humidity	0.7	< 0.01
	UVA index	–	–
	Soil Wetness	0.9	< 0.01

3.4. Two samples showed maximum quantification of withametelin and daturaolone via RP HPLC

The quantitative method developed here had been successfully applied to quantification analysis of withametelin and daturaolone in dry powders of *D. innoxia*. Based on the results of the study, the proposed method can be used easily for analysis. The quantitative results of the two compounds are shown in Table 3, Fig. 3(1) and Fig. 3(2). It appears that the distribution of withametelin is mostly found in leaves with a maximum quantified value of 5.12 ± 0.28 µg/mg dry powder when collected in June from the arid Mz region and extracted with Ea + E. During this period, earth temperature is at maximum. On contrary, the lowest humidity, soil wetness and UVA irradiance was noted. Quantity lowers down in months when the temperature falls whereas humidity and soil wetness rise. Withametelin quantity was also less in the mountainous Islamabad (I) region where soil wetness and UVA irradiance were high. Mainly, a positive correlation ($P < 0.05$) with temperature was observed. Temperature modulation is reported to cause the accumulation of alkaloids and their biological synthesis is promoted by high temperatures. Morphinane, phthalisoquinoline and benzylisoquinoline in *Papaver somniferum* was limited at low temperatures (Bernáth and Tetenyi, 1981). Similarly, the distribution of daturaolone is mostly found in fruits with a maximum quantified value of 5.18 ± 0.45 µg/m g dry powder when collected in August from the mountainous I region and extracted with Ea + E. Highest humidity and soil wetness were observed, and high UVA irradiance was noted. The quantity of daturaolone also lowers in months with a decline in humidity and soil wetness. Daturaolone quantity was less in the arid (Mz) region where soil wetness and UVA irradiance were low. Mainly, a positive correlation with soil wetness ($P < 0.01$) and humidity ($P < 0.01$) was noted. Extraction in green solvents i.e., EA: E (1:1) gave maximum results. Ethyl acetate is an environmentally benign green solvent (Häckl and Kunz, 2018). The updated GSK solvent selection guide also places it as relatively greener than most. But this does not mean that the end decision of solvent greenness is finally and definitively achieved (Byrne et al., 2016). Similarly, bio-solvents, i.e. solvents from renewable sources such as ethanol from sugar-containing feed fermentation, starch feeds and lignocellulosic feeds are used to avoid the use of fossil resources and CO_2 emissions from fossil fuels into the environment (Capello et al., 2007).

4. Conclusion

Altogether, chromatographic methods were developed for the detection and quantification of withametelin and daturaolone. The study provides evidence of the selection of the best biomass and extraction medium for the yield enhancement of withametelin and daturaolone from *Datura innoxia*. Variation in withametelin and daturaolone content was observed depending upon the plant part, geographical area, collection time (month), agroclimatology parameters and extraction medium. Withametelin can be isolated in higher yield when leaves are collected in June from the arid Muzaffargarh region and extracted with ethyl acetate + ethanol. Similarly, fruits collection from mountainous Islamabad in June can give a higher yield of daturaolone when extracted with ethyl acetate + ethanol. However, the direct and interactive contributions of each factor cannot be considered from this data. The use of a controlled environment research to determine the quantitative relationship between various parameters is proposed.

CRediT authorship contribution statement

Muhammad Waleed Baig: Methodology, Software, Validation, Investigation, Writing – original draft, Funding acquisition. **Ihsan-ul Haq:** Supervision, Resources, Project administration, Writing – review & editing. **Syeda Tayyaba Batool Kazmi:** Methodology, Funding acquisition. **Aroosa Zafar:** Methodology, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data

Background data will be provided by corresponding author upon reasonable request.

References

Baig, M.W., Fatima, H., Akhtar, N., Hussain, H., Okla, M.K., Al-Hashimi, A., Al-Qahtani, W.H., Abdelgawad, H., Haq, I.-U., 2021. Anti-inflammatory potential of daturaolone from datura innoxia mill.. in silico, in vitro and in vivo studies. Pharmaceuticals 14, 1248.

Baig, M.W., Nasir, B., Waseem, D., Majid, M., Khan, M.Z.I., Haq, I.-U., 2020. Withametelin: a biologically active withanolide in cancer, inflammation, pain and depression. Saudi Pharma. J. 28, 1526–1537.

Bernáth, J., Tetenyi, P., 1981. The effect of environmental factors on growth, development and alkaloid production of Poppy (Papaver somniferum L.): II. interaction of light and temperature. Biochem. Physiol. Pflanzen 176, 599–605.

Byrne, F.P., Jin, S., Paggiola, G., Petchey, T.H., Clark, J.H., Farmer, T.J., Hunt, A.J., Robert mcelroy, C., Sherwood, J., 2016. Tools and techniques for solvent selection: green solvent selection guides. Sustain. Chem. Process. 4, 1–24.

Capello, C., Fischer, U., Hungerbühler, K., 2007. What is a green solvent? a comprehensive framework for the environmental assessment of solvents. Green Chem. 9, 927–934.

Ferenczi-Fodor, K., Végh, Z., Renger, B., 2006. Thin-layer chromatography in testing the purity of pharmaceuticals. TrAC, Trends Anal. Chem. 25, 778–789.

GUIDELINE, I. H. T. 2005. Validation of analytical procedures: text and methodology. *Q2 (R1),* 1**,** 05

Häckl, K., Kunz, W., 2018. Some aspects of green solvents. C. R. Chim. 21, 572–580.

Kaale, E., Risha, P., Layloff, T., 2011. TLC for pharmaceutical analysis in resource limited countries. J. Chromatogr. A 1218, 2732–2736.

KANU, A. B. 2021. Recent developments in sample preparation techniques combined with high-performance liquid chromatography: A critical review. *Journal of Chromatography A,* 1654**,** 462444

Landim, L.P., Feitoza, G.S., da Costa, J.G., 2013. Development and validation of a HPLC method for the quantification of three flavonoids in a crude extract of Dimorphandra gardneriana. Revista Brasileira de Farmacognosia 23, 58–64.

NASA 2022 (Accessed on 5 January, 2022).

PATIL, M. P. N. 2017. HPLC Method Development–A Review. *Journal of Pharmaceutical Research and Education,* 1**,** 243-260

PYKA, A. 2014. Detection progress of selected drugs in TLC. *BioMed research international,* 2014.

Sarker, S.D., Nahar, L., 2015. Applications of high performance liquid chromatography in the analysis of herbal products. Elsevier, Evidence-Based Validation of Herbal Medicine.

Seo, J.-H., Kim, J.-E., Shim, J.-H., Yoon, G., Bang, M., Bae, C.-S., Lee, K.-J., Park, D.-H., Cho, S.-S., 2016. HPLC analysis, optimization of extraction conditions and biological evaluation of Corylopsis coreana Uyeki Flos. Molecules 21, 94.

Sulastri, A., Maulana, Y.E., Amaliya, A., Sukrasno, S., Soemardji, A. A., 2020. Development and validation of a RP-HPLC method for a simultaneous analysis of quercetin and ascorbic acid in psidium guajava fruit extract at different ripening stages. J. Eng. Sci. Technol. 15, 3615–3624.

Verma, N., Shukla, S., 2015. Impact of various factors responsible for fluctuation in plant secondary metabolites. J. Appl. Res. Med. Aromat. Plants 2, 105–113.

WOLFENDER, J.-L., GLAUSER, G., BOCCARD, J. & RUDAZ, S. 2009. MS-based plant metabolomic approaches for biomarker discovery. *Natural Product Communications,* 4**,** 1934578X0900401019.

Wolfender, J.-L., Marti, G., Thomas, A., Bertrand, S., 2015. Current approaches and challenges for the metabolite profiling of complex natural extracts. J. Chromatogr. A 1382, 136–164.

Yang, L., Wen, K.-S., Ruan, X., Zhao, Y.-X., Wei, F., Wang, Q., 2018. Response of plant secondary metabolites to environmental factors. Molecules 23, 762.

Yang, Y., Chen, J., Liu, Q., Ben, C.C., Todd, C.D., Shi, J., Yang, Y., Hu, X., 2012. Comparative proteomic analysis of the thermotolerant plant Portulaca oleracea acclimation to combined high temperature and humidity stress. J. Proteome Res. 11, 3605–3623.