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VARIATIONS IN BIOLOGICAL ATTRIBUTES AND PHENOLICS OF ENZYMATICALLY HYDROLYSED MEDICINAL PLANT EXTRACTS

RAHMAN QADIR, FAROOQ ANWAR*, TAHIR MEHMOOD[1], SADAF ZAHOOR AND NAUNAIN MEHMOOD[2]

Department of Chemistry, University of Sargodha, Sargodha, Pakistan

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Abstract

Major objective of this study was to appraise the variations in biological activities and phenolics of enzymatically hydrolysed medicinal plants, namely *Morus* alba (L.), *Momordica balsamina* (L.), *Capparis spinosa* (L.), *Pongamia pinnata* (L.) and *Peganum hermala* (L.) indigenous to Pothoharic region of Pakistan. Enzyme cocktails such as kemzyme dry-plus, natuzyme and zympex-014 were employed for enzyme-assisted extraction. Best antimicrobial activity was exhibited by zympex-014 produced extracts against selected strains of bacteria and fungi with inhibition zone of 18.54 and 21.45 mm, respectively. Similarly, zympex-014 produced *C. spinosa* extract (1.21%) exhibited least hemolytic activity. However, greater thrombolytic activity (51.93%) was exhibited by kemzyme dry-plus produced *M. balsamina* extract. Moreover, major phenolics detected in selected medicinal plants using RP-HPLC were gallic acid (272 µg/g), quercitin (269 µg/g), benzoic acid (184 µg/g), vanillic acid (100 µg/g) and cinnamic acid (68 µg/g). Overall, the above results revealed that enzymatic pre-treatment facilitated in the liberation of bound phenolic moieties from selected medicinal plants and thus improved their biological attributes.

The use of wild plants for the treatment of different health disorders has been an ancient tradition in the folk system of Indo-Pak medicines (Muhammad *et al*. 2015). Even though allopathic medicines are extensively employed for medication, however, the treatment of different diseases and health problems using wild medicinal plants products is still a common practice, especially in rural communities of Pakistan. Pakistan is overwhelmingly an agricultural country and is blessed with diverse agro-ecological zones. In this regard, the Pothohar Plateau, forming the northern part of Punjab, is especially important due to its rich and valued biodiversity and flora of wild medicinal plants (Ahmad *et al*. 2016).

Natural antioxidants such as phenolics, found in various plant products may lessen the risks of various pathologies and improve general human health (Shahid *et al*. 2007, Zahoor *et al*. 2016). However, selection of an appropriate procedure is a key step for extraction of optimum amount of plant phenolics with potent biological activities (Sajid *et al*. 2012). To cope with the challenges of less efficient conventional extraction methods and in line with the green chemistry principles, a recent development in the area of plant bioactives extraction is the use of enzyme-assisted maceration (enzymatic pre-treatment) to facilitate and improve the recovery of phenolics from the compact and complex plant cell wall structures into the extraction solvent (Anwar *et al*. 2013).

The present study was mainly designed to evaluate the effect of different enzyme pretreated macerations on the yield, and biological attributes of phenolic components extracted from selected medicinal plants wildly growing in the Pothoharic region. The selected medicinal plants have not yet been explored for the phenolic bioactives profiling with the primary aim to link up and establish scientific basis for their traditional folk medicinal uses. Hence, this gap of scientific information prompts the need to screen the selected wild medicinal plants growing in and around

*Author for correspondence: <fqanwar@yahoo.com>. [1]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences - UVAS, Lahore, Pakistan [2]Department of Zoology, University of Sargodha, Sargodha, Pakistan.

Pothoharic region for their phenolic antioxidants/antimicrobial profiling and biological bioactivities.

Commercial enzyme mixtures (kemzyme dry-plus, natuzyme and zympex-014) were procured from local suppliers. All other chemicals (analytical grade) used in different experiments were purchased from Sigma-Aldrich Chemical Corporation, Germany.

Three different samples of the selected parts of medicinal plants such as leaves of *M. alba, P. pinnata, P. hermala,* and fruits of *M. balsamina* and *C. spinosa* were collected from Pothoharic region of Punjab, Pakistan. After ambient drying, the materials were ground into fine powder. For enzymatic pre-treatment/hydrolysis, the material was mixed with 15 ml of phosphate buffer (6 - 9 pH) in a flask and blended with selected enzyme complex (0.5 - 6.5 g) for 30 - 90 min. After heating at 100°C for 10 min to deactivate the enzyme, the treated material was extracted in orbital shaker with 100 ml 80% aqueous methanol and filtered (0.22 µm) under pressure (Heidolph, Germany). After removal of excess solvent via distillation under reduced pressure using rotary evaporator, the crude concentrated extracts were obtained (Qadir *et al.* 2019).

Disc diffusion method was employed to evaluate antimicrobial activity of the extracts against *Escherichia coli, Bacillus subtilis, Aspergillus flavus* and *Fusarium oxysporum* (NCCLS, 1997). Rifamycin and fluconazole **(**10 mg/ml, 10 µg/disc**)** were used as positive references for bacteria and fungi, respectively.

By employing spectrophotometeric method, *in vitro* haemolytic activity was examined by taking measurement at 540 nm (Qadir *et al.* 2018). Phosphate buffer saline and Triton X-100 were used as negative and positive hemolytic controls, respectively.

Appraisal of antithrombotic activity was made by procedure described by Prasad *et al*. (2006) with slight modifications. Streptokinase (SK) was used as a positive control.

The most potent i.e., zympex-014 assisted extract was analysed by reversed phase high performance liquid chromatography (RP-HPLC) with little modifications (Abadio *et al*. 2012).

Antibacterial potential of the selected medicinal plant extracts was evaluated following the measurement of zone of inhibition (ZOI). The results indicated that *B. subtilis* was the most sensitive microorganism showing greater zone of inhibition, from 12 - 18 mm when exposed to the leaf extracts of *P. pinnata* among others (Table 1). However, *E. coli* showed better results upon exposure to the *M. alba* leaf extracts by showing zone of inhibition in the range of 12 - 16 mm. On the other side, the fruit extracts of *M. balsamina* exhibited lowest ZOI from the edges of paper disk (4 - 8 mm and 5 - 8 mm) against *E. coli* and *B. subtilis*, respectively. However, *C. spinosa* extract exhibited value in the range of 9 - 14 mm and 8 - 12 mm against *E. coli* and *B. subtilis,* respectively. Nevertheless, these activities were apparently lower than the drug (Rifamycin) used as positive control.

In case of antifungal activity, maximum ZOI was observed against *A. flavus* (15 - 21 mm) and *F. oxysporum* (16 - 19 mm) by *M. alba* extract. On the other side, lowest inhibition zones were expressed in case of *A. flavus* and *F. oxysporum* by *M. balsamina* extract ranging from 6 - 11 mm and 5 - 8 mm, respectively (Table 2). Overall, the activity exhibited by zympex –014 extract was relatively higher in all cases except in *C. spinosa*, where kemzyme dry-plus extract showed superior activity.

Previous results reported by Bajpai *et al*. (2009), Wang *et al*. (2012) and Ines *et al.* (2014) confirm the present findings and support that enzyme-assisted extraction enhanced the bioactives, thereby resulting potent biological activities of plant extracts.

Table 1. Antibacterial activity of selected medicinal plants extracts.

Sample	Antibacterial activity (Zone of inhibition in mm)					
	Bacterial strains	Methanolic extract	Kemzyme dry-plus extract	Natuzyme extract	Zympex-014 extract	Standard drug (Rifamycin)
Morus alba	*E. coli*	$12.45 \pm 0.55^{d}{}_{AB}$	$14.12 \pm 0.65^{c}{}_{A}$	$15.34 \pm 0.43^{bc}{}_{A}$	$16.56 \pm 0.56^{b}{}_{B}$	$24.65^{a}{}_{A}$
	B. subtilis	$13.76 \pm 0.45^{c}{}_{A}$	$14.23 \pm 0.34^{bc}{}_{A}$	$14.63 \pm 0.36^{b}{}_{A}$	$14.87 \pm 0.43^{bc}{}_{C}$	$21.32^{a}{}_{B}$
Pangonia pinnata	*E. coli*	8.55 ± 0.79	$12.42 \pm 0.87^{c}{}_{B}$	$15.60 \pm 0.53^{b}{}_{A}$	$15.73 \pm 0.65^{b}{}_{B}$	$24.65^{a}{}_{A}$
	B. subtilis	$11.24 \pm 1.01^{e}{}_{B}$	$14.21 \pm 1.04^{d}{}_{A}$	$16.63 \pm 1.04^{c}{}_{A}$	$18.54 \pm 0.37^{b}{}_{A}$	$21.32^{a}{}_{B}$
Peganum hermala	*E. coli*	$10.56 \pm 0.89^{d}{}_{BC}$	$13.32 \pm 0.88^{bc}{}_{AB}$	$12.10 \pm 0.64^{c}{}_{C}$	$14.23 \pm 0.51^{b}{}_{C}$	$24.65^{a}{}_{A}$
	B. subtilis	$9.56 \pm 0.78^{d}{}_{C}$	$12.62 \pm 0.76^{c}{}_{B}$	$14.34 \pm 0.56^{b}{}_{B}$	$16.11 \pm 0.63^{b}{}_{B}$	$21.32^{a}{}_{B}$
Momordica balsamina	*E. coli*	$4.87 \pm 0.34^{c}{}_{D}$	$4.43 \pm 0.21^{c}{}_{D}$	$6.54 \pm 0.26^{c}{}_{F}$	$8.34 \pm 0.11^{b}{}_{E}$	$24.65^{a}{}_{A}$
	B. subtilis	$5.67 \pm 0.32^{c}{}_{D}$	$6.12 \pm 0.58^{c}{}_{C}$	$8.17 \pm 0.38^{b}{}_{E}$	$7.45 \pm 0.16^{b}{}_{E}$	$21.32^{a}{}_{B}$
Capparis spinosa	*E. coli*	$9.68 \pm 0.65^{c}{}_{C}$	$14.71 \pm 1.01^{b}{}_{A}$	$10.83 \pm 0.44^{c}{}_{D}$	$13.65 \pm 0.41^{b}{}_{C}$	$24.65^{a}{}_{A}$
	B. subtilis	$8.56 \pm 0.45^{c}{}_{C}$	$12.67 \pm 0.81^{b}{}_{B}$	$11.23 \pm 1.21^{b}{}_{CD}$	$10.44 \pm 0.53^{bc}{}_{D}$	$21.32^{a}{}_{B}$

Values (Mean ± Sd) are average of three different samples of each plant species, analyzed individually in triplicate ($p < 0.05$). Different subscript letters within the same column indicate significant ($p < 0.05$) differences of means among the extraction medium; Superscript letters within the same row indicate significant ($p < 0.05$) differences of means among different bacterial strains analysed

Table 2. Antifungal activity of selected medicinal plants extracts.

Sample	Antifungal activity (Zone of inhibition in mm)					
	Fungal strains	Methanolic extract	Kemzyme dry-plus extract	Natuzyme extract	Zympex-014 extract	Standard drug (Fllucanozole)
Morus alba	*A. flavus*	$15.45 \pm 0.21^{b}{}_{A}$	$20.34 \pm 0.31^{b}{}_{A}$	$20.78 \pm 0.26^{b}{}_{A}$	$21.45 \pm 0.32^{b}{}_{A}$	$24.45^{a}{}_{A}$
	F. oxysporum	$16.56 \pm 0.42^{c}{}_{B}$	$17.98 \pm 0.34^{b}{}_{B}$	$18.21 \pm 0.51^{bc}{}_{B}$	$19.56 \pm 0.57^{a}{}_{A}$	$20.34^{a}{}_{B}$
Pangonia pinnata	*A. flavus*	$13.55 \pm 0.17^{d}{}_{C}$	$15.44 \pm 0.27^{bc}{}_{C}$	$14.87 \pm 0.55^{c}{}_{C}$	$16.76 \pm 0.67^{b}{}_{B}$	$24.45^{a}{}_{A}$
	F. oxysporum	$12.32 \pm 0.09^{bc}{}_{CD}$	$13.35 \pm 0.35^{b}{}_{D}$	$12.78 \pm 0.23^{bc}{}_{D}$	13.45 ± 0.52^{b}	$20.34^{a}{}_{B}$
Peganum hermala	*A. flavus*	$11.43 \pm 0.33^{c}{}_{D}$	$12.32 \pm 0.19^{c}{}_{DE}$	$15.76 \pm 0.46^{b}{}_{C}$	$15.97 \pm 0.32^{b}{}_{BC}$	$24.45^{a}{}_{A}$
	F.oxysporum	$10.56 \pm 0.07^{c}{}_{DE}$	$13.45 \pm 0.62^{b}{}_{D}$	$14.37 \pm 0.77^{b}{}_{C}$	$14.87 \pm 1.02^{b}{}_{C}$	$20.34^{a}{}_{B}$
Momordica balsamina	*A. flavus*	$6.87 \pm 0.33^{c}{}_{F}$	$10.65 \pm 0.45^{b}{}_{F}$	$7.98 \pm 0.12^{c}{}_{F}$	$11.20 \pm 0.74^{b}{}_{E}$	$24.45^{a}{}_{A}$
	F. oxysporum	$6.57 \pm 0.07^{c}{}_{F}$	$7.12 \pm 0.09^{bc}{}_{G}$	$8.37 \pm 0.15^{b}{}_{E}$	$6.45 \pm 0.11^{c}{}_{F}$	$20.34^{a}{}_{B}$
Capparis spinosa	*A. flavus*	$11.47 \pm 0.42^{bc}{}_{D}$	$12.45 \pm 0.23^{b}{}_{DE}$	$11.09 \pm 0.53^{bc}{}_{CD}$	$10.65 \pm 0.62^{c}{}_{E}$	$24.45^{a}{}_{A}$
	F. oxysporum	$9.83 \pm 0.32^{d}{}_{E}$	$14.34 \pm 0.27^{c}{}_{E}$	$10.56 \pm 0.52^{d}{}_{D}$	$13.05 \pm 0.68^{b}{}_{C}$	$20.34^{a}{}_{B}$

Values (Mean ± Sd) are average for three different samples of each plant species, analyzed individually in triplicate ($p < 0.05$). Different subscript letters within the same column indicate significant ($p < 0.05$) differences of means among the extraction medium; Superscript letters within the same row indicate significant ($p < 0.05$) differences of means among different fungal strains analyzed

Table 3. Hemolytic activity and thrombolytic activity of selected medicinal plants extracts.

Sample	Hemolytic activity				Thrombolytic activity			
	Methanolic extract	Kemzyme dry-plus	Natuzyme	Zympex-014	Methanolic extract	Kemzyme dry-plus	Natuzyme	Zympex-014
M. alba	$6.43 \pm 0.43^{b}{}_{B}$	$3.84 \pm 0.23^{c}{}_{C}$	$4.52 \pm 0.57^{a}{}_{A}$	$5.34 \pm 0.23^{b}{}_{A}$	$12.54 \pm 0.65^{c}{}_{A}$	$13.67 \pm 0.59^{bc}{}_{A}$	$16.24 \pm 1.02^{a}{}_{A}$	$14.53 \pm 0.97^{b}{}_{A}$
P. pinnata	$7.91 \pm 0.21^{b}{}_{C}$	$4.23 \pm 0.64^{a}{}_{A}$	$2.83 \pm 0.21^{c}{}_{C}$	$3.54 \pm 0.34^{b}{}_{B}$	$15.51 \pm 0.87^{c}{}_{B}$	$18.84 \pm 1.27^{b}{}_{B}$	$25.46 \pm 1.43^{a}{}_{B}$	$26.34 \pm 1.25^{a}{}_{B}$
P. hermala	$8.45 \pm 0.52^{a}{}_{A}$	$6.95 \pm 0.51^{a}{}_{B}$	$3.96 \pm 0.34^{b}{}_{BC}$	$3.24 \pm 0.42^{c}{}_{C}$	$22.12 \pm 0.95^{d}{}_{C}$	$27.18 \pm 1.04^{c}{}_{C}$	$30.08 \pm 1.36^{b}{}_{C}$	$32.34 \pm 1.46^{a}{}_{C}$
M. balsamina	$7.46 \pm 0.42^{b}{}_{B}$	$5.96 \pm 0.34^{a}{}_{A}$	$4.35 \pm 0.31^{d}{}_{B}$	$3.35 \pm 0.19^{c}{}_{B}$	$33.07 \pm 0.98^{c}{}_{D}$	$51.93 \pm 1.21^{a}{}_{D}$	$48.23 \pm 1.45^{b}{}_{D}$	$50.34 \pm 1.19^{a}{}_{D}$
C. spinosa	$3.66 \pm 0.12^{a}{}_{C}$	$1.79 \pm 0.09^{ab}{}_{D}$	$1.34 \pm 0.21^{b}{}_{C}$	$1.21 \pm 0.08^{ab}{}_{D}$	$28.98 \pm 1.16^{c}{}_{E}$	$32.82 \pm 1.23^{b}{}_{E}$	$47.24 \pm 1.05^{a}{}_{D}$	$45.25 \pm 1.11^{b}{}_{E}$
PBS	0.87				Water	3.56		
Triton X-100	96.45				Streptokinase	81.54		

The hemolytic activity of selected medicinal plant extracts such as *M. alba, P. pinnata, P. hermala, M. balsmaina* and *C. spinosa* was noted to be in the range of 3.84 - 5.34, 2.83 - 8.23, 3.24 - 6.95, 3.35 - 7.96 and 1.34 - 2.34%, respectively (Table 3). Overall, the highest hemolytic activity was observed for *P. pinnata* extract (8.23%), recovered with kemzyme dry-plus, while the lowest activity was exhibited by the fruit extract of *C. spinosa* (1.21%) which was extracted with zympex-014. Lower hemolytic activity (<5%) directly relates to lower cytotoxic effects of natural products and this supports the potential uses of under study plant extracts in nutra-pharmaceutical industries (Qadir *et al*. 2018, Mehmood *et al*. 2019).

The results of the present study, showing that enzyme-assisted plant extracts have good activity relative to the control, can be supported by the previous findings of Sabah *et al*. (2012), Milad *et al*. (2014) and Suthin *et al*. (2014) who investigated enzyme-assisted extraction to be an effective process for optimal recovery of bioactive extracts.

Thrombolytic activity of the enzyme-assisted extracts obtained from the selected medicinal plants such as *M. alba, P. pinnata, P. hermala, M. balsamina* and *C. spinosa* was found to be in the range of 12 - 16, 15 - 26, 22 - 32, 33 - 51 and 28 - 47%, respectively (Table 3). Streptokinase was used as a positive control (81.54%). The highest thrombolytic activity was observed for kemzye dry-plus and zympex-014 based extracts of *M. balsamina* (51.93 and 50.34%) while the lowest for methanolic extracts of *M. alba* and *P. pinnata* (12.54 and 15.51%). In case of *C. spinosa*, the maximum thrombolytic activity was observed for natuzyme-based extract (47.24%) while the minimum for control was methanolic extract (28.98%).

Based on the results of thrombolytic activity, it can be conferred that zympex-014 based *C. spinosa* and *M. balsamina* extracts exhibited better activity compared to other tested extracts. As enzyme complexes have different extracting power to recover phenolic contents, therefore the difference in thrombolytic activity among different enzyme complexes might be related to their varying composition in addition to bioactives composition of plant.

Table 4. HPLC characterization of phenolics in selected medicinal plants (zympex-014-assisted plant extracts).

Sl. No.	Phenolics	Phenolics concentration (µg/g)				
		M. alba	*P. pinnata*	*P. hermala*	*M. balsamina*	*C. spinosa*
1	Quercetin	102 ± 3.51	143 ± 5.71	132 ± 3.51	169 ± 5.33	175 ± 2.71
2	Gallic acid	120 ± 5.12	164 ± 6.52	106 ± 0.92	107 ± 1.84	95 ± 3.12
3	Caffeic acid	ND	ND	20 ± 0.43	ND	ND
4	Vanillic acid	34 ± 2.33	41 ± 1.55	ND	ND	ND
5	Benzoic acid	12 ± 2.44	ND	13 ± 1.54	14 ± 2.32	11 ± 0.82
6	*p*-coumaric acid	17 ± 0.55	ND	ND	ND	ND
7	Chlorogenic acid	ND	28 ± 0.78	ND	ND	30 ± 1.63
8	*m*-coumaric acid	11 ± 1.34	ND	19 ± 0.46	13 ± 0.51	14 ± 0.94
9	Ferulic acid	ND	ND	ND	36 ± 0.89	ND
10	Cinnamic acid	35 ± 2.36	25 ± 3.17	ND	ND	28 ± 1.9
11	Sinapic acid	ND	ND	19 ± 1.17	13 ± 0.78	ND

ND = not detected.

The most effective i.e., zympex-014-assisted plant extract was subjected to phenolics analysis by RP-HPLC that revealed the presence of a pool of phenolics (Table 4). Major phenolics detected were gallic acid, benzoic acid, quercetin, vanillic acid and cinnamic acid. The amount of gallic

acid varied over a broad range, 46 - 271 µg/g. Other important phenolic compounds such as caffeic acid and ferulic acid were present only in *P. hermala* and *M. balsamina*, respectively.

Previous HPLC results of *M. alba* and *P. pinnata* leaf extracts demonstrated the presence of *p*-hydroxybenzoic acid, vanillic acid, chlorogenic acid, *m*-coumaric acid (Ayaz *et al*. 2010, Sajid *et al*. 2012). Likewise, various phenolics were also identified and isolated from the methanolic extracts of *C. spinosa* (Yu *et al*. 2006, Tehseen *et al*. 2015). Moreover, the identified phenolic compounds are known to have antioxidant and other useful biological properties and this supports the medicinal and health promoting role of these phytochemicals.

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