SUPPLEMENTARY MATERIAL

Chemical composition and insecticidal activities of essential oils against diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae)

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Abstract

Five himalayan plants namely *Acorus calamus*, *Cedrus deodara*, *Aegle marmelos*, *Tagetes minuta* and *Murraya koenigii* were used for the extraction of essential oils through hydro distillation and the major volatile constituents as identified by GC and GC-MS techniques were beta asarone (91.1%), beta himachalene (45.8%), limonene (59.5%), Z-ocimene (37.9%) and alpha pinene (54.2%), respectively. Essential oils were tested for their insecticidal properties against larvae of diamond back moth, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae). Results showed that *A. calamus* was most toxic ($LC_{50} = 0.29$ mg mL⁻¹) to *P. xylostella* followed by *C. deodara* ($LC_{50} = 1.08$ mg mL⁻¹) and *M. koenigii* ($LC_{50} = 1.93$ mg mL⁻¹) via residual toxicity bioassay. Percent feeding deterrence index and growth inhibition was significantly higher in *A. calamus* (42.20 and 68.55 respectively) followed by *C. deodara* (35.41 and 52.47). In repellent activity studies, *C. deodara* showed high repellence (64.76%) followed by *A. calamus* (55.05%).

Keywords: Essential oils; chemical composition; residual toxicity; antifeedant; repellence

Experimental

Plant material and extraction of essential oil

The plant materials of *Tagetes minuta* (TM) (Bharmour, Himachal Pradesh, India, October 2012), *Aegle marmelos* (AM) (Panchrukhi, Himachal Pradesh, India, August 2012), *Murraya koenigii* (MK) (Panchrukhi, Himachal Pradesh, India, July 2009), *Acorus calamus* (AC) (Panchrukhi, Himachal Pradesh, India, April 2012) and *Cedrus deodara* (CD) wood chips (Mandi, Himachal Pradesh, India, May 2009) were collected from different locations in Himachal Pradesh, India. The plant material was authenticated and voucher specimens were deposited in the herbarium of CSIR-IHBT, Palampur (H.P.). The voucher number for AM, MK, AC and CD were PLP 17734, 17731, 17730 and 5969, respectively). All the essential oils were obtained using Clevenger type apparatus through hydro distillation. Each essential oil sample was dried over anhydrous sodium sulphate and placed at low temperature until used for further analysis.

Gas chromatography analysis and quantification

The chemicals composition of the oils were carried out by gas chromatography (GC) on Shimadzu GC 2010 equipped with DB-5 (J&W Scientific, Folsom, CA, USA) fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) and FID detector. The GC oven temperature program was as follows, 40°C (initial temperature) held for 4 min and then at a rate of 4°C/min to 220°C and held for 15 min. Injector temperature, 250°C, detector temperature, 250°C, injection mode split. Carrier gas was helium at column flow rate of 1.24 mL/min (87.9 kPa).

Retention indices (RI) of the sample components and authentic compounds were determined on the basis of homologous *n*-alkane hydrocarbons (C₉–C₂₄) under the same conditions. The quantitative composition was obtained by peak area normalization and the response factor for each component was considered equal to 1.

GC/MS analysis and identification

The gas chromatography/mass spectrometry (GC/MS) analysis of oils was conducted using a Shimadzu QP 2010 using a DB-5 (J&W Scientific, Folsom, CA, USA) capillary column (30 m × 0.25 mm i.d., 0.25 µm thickness). The GC oven temperature was 40°C for 4 min and then to 220°C at 4°C/min and held for 15 min. Injector temperature, 250°C, Interface temperature, 250°C, acquisition mass range, 800–50 amu, ionization energy, 70 eV. Helium was used as carrier gas.

Compounds were identified by using library search of National Institute of Standards and Technology (NIST) database (Stein, 1990) as well as by comparing their RI and mass spectral fragmentation pattern with those reported in literature (Adams, 2007).

Insecticidal activity of essential oils against diamondback moth, *Plutella xylostella*

Test insect

P. xylostella used for the experimental study was collected from infested cabbage (*Brassica oleracea* L.) field and reared under laboratory conditions on mustard, *Brassica juncea* (L.) seedlings for more than 50 generations at constant temperature (25 ± 2°C), relative humidity (60 ± 5%) and photoperiod (16:8 L: D). Neonate uniform second/third instar larvae were used for testing insecticidal activities.

Preliminary screening of essential oils against *P. xylostella*

Preliminary screening of essential oils at higher concentrations (10 and 5 mg mL⁻¹) was tested for their toxicity against second instar larvae of *P. xylostella*. Based on preliminary screening results, five concentrations/dosages were fixed for each essential oil and tested in the main experiments.

Residual toxicity of essential oils

Residual toxicity of essential oils was tested following leaf dip bioassay (Park et al. 2002) against second instar larvae of *P. xylostella*. Hundred milligrams of the test samples (essential oils) were dissolved in 10

mL of 0.05 percent Tritone (SD Fine Chemicals Limited, www.sdfine.com) in water and then ultrasonicated for complete dissolution. Five concentrations (10, 5, 2.5, 1.25 and 0.25 mg mL⁻¹) of test solutions were prepared from stock solutions by serial dilution from the solution of higher concentration for dose response bioassay. The prepared concentrations were poured in glass petri dishes. Fresh mustard leaf discs (4.2 cm diameter) were cut from mustard leaves grown under greenhouse conditions without any insecticide spray. Leaf discs were dipped in essential oil emulsions for 10 seconds and then allowed to air dry at room temperature. For control, leaf disks were dipped in distilled water containing 0.05 percent Tritone. Treated leaf discs were placed individually into glass Petri dishes with moistened filter paper to prevent desiccation. Ten larvae starved for 4 h were transferred onto the treated leaf discs in Petri dish (6 cm diameter) and then sealed with para film and kept in the laboratory conditions at 25 ± 2 °C temperature, 60 ± 5 per cent relative humidity and a photoperiod of 16:8 (L: D) for observations. Three replicates of 10 larvae per concentration were maintained. The observation on mortality was recorded at 24 h interval.

Repellent activity of essential oils

The repellent activities of essential oils were tested at three concentrations (10, 5 and 2.5 mg mL⁻¹) against larvae of *P. xylostella* by choice test. Test solutions/concentrations for repellent activity were prepared following similar procedure followed in residual toxicity experiment. Mustard leaf discs (4–5 cm diameter) were cut from mustard leaves and dipped into essential oil emulsions for 10 seconds and then allowed to air dry. The treated leaf discs were placed alternatively with untreated leaf discs in a circular fashion at equal distance on a drawing sheet (72 cm × 56 cm). Twenty five third instar larvae of *P. xylostella* were released freely at the centre of the drawing sheet. Larvae were allowed to settle on the leaf discs of their choice for 15 min. The observations were made on the number of larvae settled on treated and untreated leaf discs. Each treatment was replicated seven times. Percent repellent activity was calculated by using formula.

$$\text{Percent repellence} = \frac{\text{No. of larvae settled on untreated leaf discs}}{\text{Total number of larvae released}} \times 100$$

Feeding deterrent activity of essential oils

The antifeedant activities of essential oils were tested at three concentrations (10, 5 and 2.5 mg mL⁻¹) against third instar larvae of *P. xylostella*. The test solutions/concentrations for feeding deterrent activity were prepared following the procedure followed in residual toxicity experiment. The leaf discs of equal dimensions were prepared from mustard leaves. The area of mustard leaf discs were measured prior to feeding/release and 48 h post feeding. Leaf discs were dipped in essential oil emulsions for 10 seconds and then allowed to air dry at room temperature. For control, leaf disks were dipped in distilled water containing 0.05 percent Tritone. The leaf discs petioles were wrapped with wet cotton swab to delay the desiccation. The treated leaf discs were placed individually into glass Petri dishes. 4-5 h starved third

1 instar larvae were transferred into the Petri dish containing treated leaf discs and then sealed with para film
 2 and kept in the laboratory conditions. Each treatment was replicated ten times with two larvae in each
 3 replication. The observations on the leaf area consumed by each set of larvae were measured after 48 h of
 4 feeding using WinDIAS Image Analysis System (Delta-T Devices Ltd., UK). The feeding deterrence index
 5 (FDI) was calculated using the formula (Akthar et al. 2008) given below.

$$\text{Feeding Deterrence Index} = \frac{\text{Leaf area consumed in control} - \text{leaf area consumed in treatment}}{\text{Leaf area consumed in control}}$$

6 ***Growth inhibition activity of essential oils***

7 Preparation of test concentrations, exposure and maintenance of the insects for the growth inhibition test
 8 was same as in the feeding deterrent activity above. Prior to exposure, all the larvae tested were
 9 individually weighed. Each treatment group consisted of ten larvae, and each treatment was repeated 10
 10 times. The growth process of the larvae was recorded after 48 h and growth inhibition rate (GIR) was
 11 calculated according to the formula (Guo et al. 2014).

$$\text{Growth Inhibition Rate (GIR)} = \frac{\text{Weight of larvae in control} - \text{Weight of larvae in treatment}}{\text{Weight of larvae in control}} \times 100$$

12

13 ***Antifeedant activity w.r. to reduction in weight gain of *P. xylostella* larvae to essential oils***

14 Preparation of test concentrations, exposure and maintenance of the insects for the reduction in the weight
 15 experiment is same as in the feeding deterrent activity above. The reduction in the weight gain of larvae
 16 that fed on treated leaves compared to the weight gain in untreated larvae was recorded after 48 h. The
 17 percent reduction in weight (PRW) calculated by using the following formula.

$$\text{Percent reduction in weight gain} = \frac{\text{Weight gain in control} - \text{Weight gain in treatment}}{\text{Weight gain in control}} \times 100$$

18 ***Statistical analysis***

19 Residual toxicity data from all bioassays were corrected for control mortality using Abbott formula (Abbott
 20 1925). The median lethal concentration (LC₅₀) and their corresponding 95 per cent confidence intervals
 21 were determined following probit analysis (Finney 1971) and SPSS 10.00 statistical tool. The data on
 22 percent feeding deterrent index (FDI), per cent reduction in weight gain and per cent growth inhibition, two

factor (Essential oils and concentrations) ANOVA was done by using SPSS 10.00 statistical tool and compared each of the properties between oils.

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Table S1. Phytochemical constituents of essential oils obtained from different plant material.

Sr. No.	Components	RI ^a	RI ^b	AC	CD	TM	AM	MK	Mode of identification
Components (%)									
1	α -Thujene	931	930	-	-	-	-	0.7	MS, RI
2	α -Pinene	939	938	-	-	0.8	4.7	54.2	MS, RI
3	Camphene	953	954	-	-	-	0.1	0.6	MS, RI
4	Sabinene	976	977	-	-	0.2	0.3	13.7	MS, RI
5	β -Pinene	980	981	-	-	-	-	8.8	MS, RI
6	Myrcene	991	992	-	-	-	1.7	0.8	MS, RI
7	α -Phellandrene	1005	1008	-	-	-	14.8	-	MS, RI
8	α -Terpinene	1018	1020	-	-	-	-	0.2	MS, RI
9	<i>p</i> -Cymene	1026	1029	-	-	-	-	-	MS, RI
10	β -Phellandrene	1031	1029	-	-	-	5.6	2.3	MS, RI
11	Limonene	1031	1033	-	-	1.9	59.5	4.5	MS, RI
12	Z-Ocimene	1040	1042	0.2	-	37.9	1.6	0.1	MS, RI
13	<i>E</i> -Ocimene	1050	1052	-	-	-	5.4	-	MS, RI
14	Dihydrotagetone	1054	1059	-	-	12.7	-	-	MS, RI
15	Terpinolene	1088	1089	-	-	-	0.1	0.3	MS, RI
16	Linalool	1098	1105	-	-	-	0.4	0.2	MS, RI
17	allo-Ocimene	1129	1131	-	-	0.5	-	-	MS, RI
18	<i>cis</i> -Limonene oxide	1134	1136	-	-	-	0.1	-	MS, RI
19	<i>E</i> -Myroxide	1142	1147	-	-	0.3	-	-	MS, RI
20	<i>E</i> -Tagetone	1146	1151	-	-	1.4	-	-	MS, RI
21	Z-Tagetone	1153	1159	-	-	11.8	-	-	MS, RI
22	4-Terpineol	1177	1172	-	-	-	0.1	4.3	MS, RI
23	Z-Ocimenone	1231	1238	-	-	5.4	-	-	MS, RI
24	<i>E</i> -Ocimenone	1239	1246	-	-	11.4	-	-	MS, RI
25	Bornyl acetate	1285	1290	-	-	-	-	0.7	MS, RI
26	β -Bourbonene	1384	1388	-	-	-	-	0.1	MS, RI
27	β -Elemene	1391	1393	t	-	-	0.1	0.3	MS, RI
28	β -Caryophyllene	1418	1425	-	-	0.4	0.5	3.1	MS, RI
29	<i>trans</i> - α -Bergamotene	1436	1438	-	0.1	-	-	-	MS, RI
30	Vestitenone	1443	1451	-	0.3	-	-	-	MS, RI
31	α -Himachalene	1447	1454	-	15.8	-	-	-	MS, RI

32	α -Humulene	1454	1455	-	-	0.2	0.2	0.7	MS, RI
33	β -Farnesene	1458	1466	-	0.3	-	-	-	MS, RI
34	γ -Himachalene	1476	1485	-	10.4	-	-	-	MS, RI
35	Germacrene-D	1480	1489	-	1.9	0.3	-	-	MS, RI
36	Bicyclogermacrene	1494	1500	-	-	0.9	-	-	MS, RI
37	β -Himachalene	1499	1508	-	45.8	-	-	-	MS, RI
38	α -Murolene	1499	1510	0.3	-	-	-	0.3	MS, RI
39	γ -Cadinene	1513	1517	0.7	-	-	-	-	MS, RI
40	δ -Cadinene	1524	1524	-	-	-	-	0.1	MS, RI
41	Kessane	1528	1525	0.2	-	-	-	-	MS, RI
42	<i>trans</i> -Calomenone	1532	1533	-	1.3	-	-	-	MS, RI
43	γ -Bisabolene	1533	1541	-	0.4	-	-	-	MS, RI
44	<i>trans</i> -Sesquisabinene hydrate	1580	1574	-	0.1	-	-	-	MS, RI
45	Caryophyllene oxide	1581	1581	-	0.3	-	0.1	0.2	MS, RI
46	Humulene epoxide II	1606	1595	-	-	-	-	0.1	MS, RI
47	β -Himachalene oxide	1610	1622	-	1.6	-	-	-	MS, RI
48	β -Asarone	1622	1631	91.1	-	-	-	-	MS, RI
49	α -Asarone	1679	1684	2.6	-	-	-	-	MS, RI
50	γ -Atlantone	1701	1706	-	2.1	-	-	-	MS, RI
51	α -Atlantone	1773	1783	-	6.7	-	-	-	MS, RI
Total				95.1	87.1	86.1	95.3	96.6	
Monoterpene hydrocarbons [*]				0.2	-	41.3	93.8	86.2	
Oxygenated monoterpene [*]				-	-	43.0	0.6	4.6	
Sesquiterpene hydrocarbons [*]				1.0	74.7	1.8	0.8	4.8	
Oxygenated Sesquiterpene [*]				0.2	12.4	-	0.1	0.3	
Phenylpropanoids [*]				93.7	-	-	-	0.7	

^aRI: value of compounds in literature data (Adams, 2007).

^bRI: Retention index determined relative to *n*-alkanes (C₉-C₂₄) on the DB-5 GC column.

^{*} Percentage of compounds class in analyzed oil samples.

t= < 0.1%.

Table S2. Residual toxicity of essential oils against 2nd instar larvae of diamondback moth, *Plutella xylostella* (48 h post treatment)

Essential oils	LC ₅₀ (mg mL ⁻¹)	95% CI (mg mL ⁻¹)	Slope ± SE	Chi square	P value
<i>Acorus calamus</i>	0.39	0.27–0.44	2.29 ± 0.34	4.69	0.19
<i>Cedrus deodara</i>	1.08	9.26–1.26	5.23 ± 0.86	0.04	0.99
<i>Murraya koenigii</i>	2.98	2.29–3.95	2.03 ± 0.31	1.0	0.80
<i>Aegle marmelos</i>	8.76	6.22–15.84	1.93 ± 0.37	3.33	0.34
<i>Tagetes minuta</i>	10.15	6.18–29.63	1.29 ± 0.30	2.28	0.49

CI: Confidence limits; LC₅₀ s (Lethal concentration causing 50% mortality of test insect population) was calculated for essential oils showing > 50% mortality using probit analysis. Five concentrations were used to calculate LC₅₀ values (0.62 to 10 mg mL⁻¹ for essential oils except *Acorus* (0.062 to 1 mg mL⁻¹))

Table S3. Residual toxicity of essential oils against 2nd instar larvae of *P. xylostella* (72 h post treatment)

Essential oils	Mortality (%) 10 mg mL ⁻¹	LC ₅₀ (mg mL ⁻¹)	95% CI (mg mL ⁻¹)	Slope ± SE	Chi square	P value
<i>A. calamus</i>	100.00 a	0.29	0.21–0.33	2.43 ± 0.34	5.13	0.16
<i>C. deodara</i>	100.00 a	1.08	9.26–1.26	5.23 ± 0.86	0.04	0.99
<i>M. koenigii</i>	100.00 a	1.93	1.51–2.43	2.46 ± 0.35	2.05	0.56
<i>A. marmelos</i>	80.00 ab	4.40	3.26–6.52	1.75 ± 0.30	2.78	0.42
<i>T. minuta</i>	53.33 b	8.45	5.10–25.20	1.15 ± 0.30	0.98	0.80

CI: Confidence limits; Figures in same alphabetical letters with in column indicate significantly at par in Duncan Multiple Range Test (DMRT); LC₅₀ s (Lethal concentration causing 50% mortality of test insect population) was calculated for essential oils showing > 50% mortality using probit analysis. Five concentrations were used to calculate LC₅₀ values (0.62 to 10 10 mg mL⁻¹) for essential oils except *Acorus* (0.062 to 1 10 mg mL⁻¹)

Table S4. Repellent activity of essential oils against *P. xylostella*

Essential oils	Per cent reduction ($\pm$ SE) in weight at different concentrations after 48 h			
	10 mg mL ⁻¹	5 mg mL ⁻¹	2.5 mg mL ⁻¹	Pooled mean
<i>A. calamus</i>	73.14 $\pm$ 5.52	59.43 $\pm$ 12.53	32.57 $\pm$ 10.18	55.05 $\pm$ 1.31b
<i>C. deodara</i>	86.29 $\pm$ 3.90	65.71 $\pm$ 3.90	42.29 $\pm$ 5.59	64.76 $\pm$ 1.31a
<i>M. koenigii</i>	63.43 $\pm$ 5.38	43.43 $\pm$ 4.86	28.57 $\pm$ 7.46	45.14 $\pm$ 1.31cd
<i>A. marmelos</i>	49.14 $\pm$ 3.02	46.86 $\pm$ 3.02	28.57 $\pm$ 4.28	41.52 $\pm$ 1.31d
<i>T. minuta</i>	81.71 $\pm$ 3.90	41.71 $\pm$ 3.90	22.86 $\pm$ 3.02	48.76 $\pm$ 1.31c
Pooled mean	70.74 $\pm$ 1.01 a	51.43 $\pm$ 1.01 b	30.97 $\pm$ 1.01 c	
Oils	F _{90 4} = 49.1; p< 0.0001			
Concn.	F _{90 2} = 386.6; p< 0.0001			
Oils x Concn.	F _{90, 8} =13.15; p< 0.0001			

Figures in same alphabetical letters with in column indicate significantly at par (DMRT)

Table S5. Antifeedant activity of essential oils against third instars larvae of *P. xylostella*

Essential oils	Feeding deterrence index ($\pm$ SE) at different concentrations after 48h			
	10 mg mL ⁻¹	5 mg mL ⁻¹	2.5 mg mL ⁻¹	Pooled mean
<i>A. calamus</i>	71.42 $\pm$ 16.74	46.01 $\pm$ 27.16	9.17 $\pm$ 30.81	42.20 $\pm$ 7.05 a
<i>C. deodara</i>	59.58 $\pm$ 22.12	30.39 $\pm$ 40.93	16.23 $\pm$ 22.70	35.41 $\pm$ 7.05 ab
<i>M. koenigii</i>	31.23 $\pm$ 51.41	12.60 $\pm$ 32.48	12.23 $\pm$ 36.75	18.69 $\pm$ 7.05 bc
<i>A. marmelos</i>	24.53 $\pm$ 29.04	10.68 $\pm$ 65.54	5.87 $\pm$ 60.20	13.69 $\pm$ 7.05 c
<i>T. minuta</i>	50.64 $\pm$ 32.03	30.49 $\pm$ 18.21	20.23 $\pm$ 50.39	33.79 $\pm$ 7.05 ab
Pooled mean	47.48 $\pm$ 5.46 a	26.03 $\pm$ 5.46 b	12.75 $\pm$ 5.46 b	
Oils	F _{135, 4} = 2.91; p < 0.05			
Concn.	F _{135, 2} = 10.30; p < 0.0001			
Oils x Concn.	F _{135, 8} = 0.64; p > 0.05			

Figures in same alphabetical letters with in column indicate significantly at par (DMRT)

Table S6. Growth (feeding) inhibition of essential oils against third instars larvae of *P. xylostella*

Essential oils	% Growth inhibition ($\pm$ SE) at different concentrations after 48 h			
	10 mg mL ⁻¹	5 mg mL ⁻¹	2.5 mg mL ⁻¹	Pooled mean
<i>A. calamus</i>	82.95 $\pm$ 7.45	68.78 $\pm$ 16.20	53.92 $\pm$ 18.73	68.55 $\pm$ 4.93 a
<i>C. deodara</i>	72.35 $\pm$ 14.60	56.84 $\pm$ 30.97	28.21 $\pm$ 47.18	52.47 $\pm$ 4.93 b
<i>M. koenigii</i>	31.03 $\pm$ 14.02	28.22 $\pm$ 18.98	27.78 $\pm$ 20.12	29.01 $\pm$ 4.93 c
<i>A. marmelos</i>	22.91 $\pm$ 18.90	21.27 $\pm$ 27.70	14.32 $\pm$ 47.97	19.50 $\pm$ 4.93 c
<i>T. minuta</i>	34.48 $\pm$ 20.44	27.00 $\pm$ 30.53	4.23 $\pm$ 33.71	21.90 $\pm$ 4.93 c
Pooled mean	48.74 $\pm$ 3.82 a	40.42 $\pm$ 3.82 a	25.69 $\pm$ 3.82 b	
Oils	F _{135, 4} = 18.76: p< 0.0001			
Concn.	F _{135, 2} = 9.34: p< 0.0001			
Oils x Concn.	F _{135, 8} =1.04: p> 0.05			

Figures in same alphabetical letters within column indicate significantly at par (DMRT)

Table S7. Reductions in weight due to essential oils against third instar larvae of *P. xylostella*

Essential oils	% Reduction in weight ($\pm$ SE) at different concentrations after 48 h			
	10 mg mL ⁻¹	5 mg mL ⁻¹	2.5 mg mL ⁻¹	Pooled mean
<i>A. calamus</i>	101.10 $\pm$ 12.41	79.33 $\pm$ 19.33	59.56 $\pm$ 21.22	80.09 $\pm$ 6.30 a
<i>C. deodara</i>	121.62 $\pm$ 20.69	99.29 $\pm$ 28.69	58.34 $\pm$ 60.09	93.09 $\pm$ 6.30 a
<i>M. koenigii</i>	63.05 $\pm$ 31.02	43.27 $\pm$ 22.45	39.91 $\pm$ 30.50	48.74 $\pm$ 6.30 b
<i>A. marmelos</i>	51.51 $\pm$ 17.06	31.90 $\pm$ 31.27	12.86 $\pm$ 53.08	32.09 $\pm$ 6.30 b
<i>T. minuta</i>	80.72 $\pm$ 24.57	57.50 $\pm$ 49.85	11.60 $\pm$ 49.47	49.94 $\pm$ 6.30 b
Pooled mean	83.60 $\pm$ 4.88 a	62.26 $\pm$ 4.88 b	36.51 $\pm$ 4.88 c	
Oils	F _{135, 4} = 15.76: p< 0.0001			
Concn.	F _{135, 2} = 23.34: p< 0.0001			
Oils x Concn.	F _{135, 8} =0.93: p> 0.05			

Figures in same alphabetical letters within column indicate significantly at par (DMRT)