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Degradation of Feather Keratin by Keratinophyles for Growth Promoting Biomolecules: A Biochemical Proof

Richa Saxena*, J. N. Shirvastava[1] and Parul Saxena[2]

Dayalbagh Educational Institute, Agra- 282005

E- mail: rsaxena.0689@gmail.com

Janendra.srivastava@gmail.com

Abstract

This work is focusing on the importance of keratinolytic fungi and their property of utilizing and degrading keratin. Degradation of keratin plays a vital role in the betterment of environment as there is large accumulation of waste in the form of keratin (hairs, feather horns, wool, nail etc) is piling on the planet, therefore it is a necessary to degrade this keratin with the help of keratinolytic fungi. Specific proteases, keratinases are the enzymes involved in this process of utilization of keratin. Feathers were considered as the keratin substrate for denaturation. Keratinophilic fungi studied in this work are Chrysosporium anamorph of Rollandina vriesii, Geomyces pannorum, Microsporum fulvum and Trichophyton mentagrophytes which inhabits the property of keratin degradation. Thus this work is considered desirable in the biodegradation of keratinous substrate.

Keywords: keratinolytic, keratinophilic fungi, accumulation, dermatophytes, keratinous, substrate.

INTRODUCTION

Keratinolytic fungi are those which posses the enzymatic ability to attack and utilize keratin. Keratin is degraded by specific proteases, keratinases (Onifade et al., 1998; Wang and Shih, 1999; Gradisia et al. 2000; Sandali and Brandelli, 2000; Kim et al., 2002; Allpress et al., 2002; Longshaw et al., 2002; Yamamura et al., 2002; Gessesse et al., 2003; Singh, 2003). Keratinolysis involves action of proteolytic and sulfitolytic systems. High sulphur content is the specific character of keratin as the sulphur containing amino acids- cystine, cysteine and methionine. The ability of these fungi to invade and parasitize cornified tissues is closely associated with the utilization of keratin by enzymatic digestion. The soil inhabiting keratinophilic fungi *Aphanoascus, Arthroderma, Chrysosporium, Ctenomyces, Malbranchea* and *Nannizia* share this capacity with dermatophytes. The virulence factor is of considerable importance and keratinase produced by this fungus is the major enzyme involved with this pathogenesis process or virulence factor (Howard, 1983). Degradation of keratinous material has been studied by various workers(Kunert, 1972a, 1973, 1976a; Evan and Hose, 1975; Kushwaha and Agarwal, 1981b; Safranek and Goos, 1982; Wainwright, 1982; Deshmukh and Agarwal, 1982, 1985; Kushwaha, 1983a, 1998; Wawrzkiewicz et al., 1987; Nigam and kushwaha, 1989a, 1990a, d, 1992; Rajak et al., 1991a, 1992; Malaviya et al.,

1991, 1992a, b; Singh et al., 1996 and El Naghy et al., 1998) and it was proved that the keratinophilic fungi can grow even on hard keratin as an only source of carbon and nitrogen. The degradation of keratin in mineral medium is accompanied by alkalization of the medium. Moreover, Lal et al., (1996) studied keratin degradation by *Actinomycetes*. Noval and Nickerson(1959); Nickerson et al., (1963); Young and Smith (1975) and sinha et al.,(1991) showed the keratinolytic activity of *Actinomycetes*. Non pathogenic fast growing keratin degrading fungi have been found implicated in keratin digestion in recent days. Non-keratinophilic fungi were also found implicated in keratin decomposition (Kunert, 1995). Reports on their frequent occurrence were made by Evan and Hose (1975); Safranek and Goos (1982); Nigam and Kushwaha (1989d); Nigam and Kushwaha (1992); Kushwaha (1995) and Kushwaha and Nigam (1996) gave the biochemical evidence supporting observations that non dermatophytic fungi can attack and utilize keratinous substrates.

Denaturation of feathers as a keratin substrate will be done by Keratinolytic fungi. *Chrysosporium* anamorph of *Rollandina vriesii*, *Geomyces pannorum*, *Microsporum fulvum* and *Trichophyton mentagrophytes* are the keratinolytic fungi, considered for the enzymatic analysis of protein and amino acid – cyseine, cystine, methionine. Feathers are choosen because they have the high nutritional content of about 90% crude protein in the form of keratin. Keeping the above view in mind, it was thought desirable to work on the mechanism of biodegradation of keratin.

MATERIALS AND METHODS

ENYMATIC ANALYSIS

Chrysosporium anamorph of *Rollandina vriesii*, *Geomyces pannorum*, *Microsporum fulvum* and *Trichophyton mentagrophytes* were subjected for enzymatic studies by quantitative analysis of amino acid (cysteine, cystine and methionine). The analysis was conducted for 35 hours at an interval of 7 days consecutively, under both shake and stationary condition respectively. Amino acids (cysteine, cystine and methionine) were analyzed as follows:-

DETERMINATION OF CYSTEINE:

Cysteine was quantitatively estimated by the method given by Ramakrishna et al., (1979).

Procedure: - To 4ml of the test sample (culture filtrate), 15 ml of phosphate buffer, 2 ml of 0.2% metol and 3 ml of $K_2Cr_2O_7$ solution was added successively. Absorbance (O.D) was measured at 510 nm after 2 hours and 40 minutes.

Standard values of cysteine, using acetate buffer (pH 5.0) as a reference are : 0.337 at 4µg/ml concentration, 0.389 at 5µg/ml and 0.494 at 7µg/ml concentration.

DETERMINATION OF CYSTINE: By

Ramakrishna et al., (1979).

Procedure: -To 5 ml of the test sample (culture filtrate), add 2 ml of 5% NaCN solution. Mix and wait for 10 minutes. Now add 1 ml of 0.5% solution of 1, 2 napthaquinone-4-sodium sulphite and 5 ml of alkaline sodium sulphite solution successively with regular shaking. Wait for 30 minutes. Reddish brown color appears. Now add 1 ml of 5N NaOH and 1 ml of

alkaline sodium hyposulphite solution. Keep it for 24 hours. The absorbance (O.D) was measured at 510 nm wavelengths after 24 hours.

Standard values of cystine, using 1N HCL as a reference are: 0.163 at 1µg/ml concentration, 0.200 at 2µg/ml and 0.422 at 8µg/ml concentration.

DETERMINATION OF METHIONINE:

Methionine was analyzed by Bioling's modification of Mc Carthy and Sullivan's method (Singh, 1994).

Procedure: - To 7.5 ml of the test sample (culture filtrate), 1.5 ml of 5N NaOH, 1.5 ml of 1% glycine and 0.3 ml of 10% sodium nitroprusside solution were added successively and kept for boiling in a hot water bath at 37 – 40 degree C for 15 minutes. After boiling the samples were allowed to stand for cooling in ice water for 5 – 7 minutes. After cooling add 3 ml of 6N HCL. Shake for 1 minute and let it stand at room temperature for 15 minutes. Measure the absorbance (O.D) at 356 nm wavelengths.

Standard (O.D) values of methionine being 0.167 at 10µg/ml concentration and 0.715 at 160µg/ml concentration, using distill water as a reference.

Concentration of all of the above amino acid is determined by the formula:-

Concentration Of unknown = Optical Density of unknown conc. Of known (standard)

Optical Density of known (standard)

Results and Discussion

Enzymatic activity was carried out to analyze the amount of protein, amino acids like cysteine, cystine and methionine. Feather was selected as a keratin substrate for denaturation. Feather, the waste from the poultry industry has a high nutritional value with over 90% crude protein in the form of keratin. These microorganisms producing enzyme keratinase have been described having the ability to degrade feathers into proteins and amino acids.

The experiment was set up for 35 days and results were observed at an interval of 7 days. The highest rate of degradation of feather was observed by *Geomyces pannorum* in both shake and stationary condition.

Breakdown of disulpide linkages in feather during microbial degradation leads to the release of protein. Extracellular protein production was maximum in case of *Geomyces pannorum* (7.37µg/ml), followed by *Chrysosporium anamorph of Rollandina vriesii* (6.21µg/ml), *Trichophyton mentagrophytes* (5.95µg/ml) and *Microsporum fulvum* (5.67µg/ml) in 21 days in shake condition. The tendency of degradation showed steady increase from 7 to 21 days, followed by a declined rate of protein released during further incubation period. Stationary condition also showed the same tendency with increase in protein release up till 21 days except in case of *Microsporum fulvum*, which showed

increased rate of protein release up to 28 days and then declined by the end phase of incubation. In stationary condition also *Geomyces pannorum* (6.10µg/ml), leaded *Trichophyton mentagrophytes* (3.97µg/ml), *Chrysosporium anamorph of Rollandina vriesii* (2.76µg/ml), and *Microsporum fulvum* (2.50µg/ml).

The most distinctive character of keratin is its high cysteine content. Presence of sulphur containing amino acids relates wit high stability of keratin. Keratinophyllic fungi degrade keratin by enzymatic breakdown of cystine disulphide bonds leading to the release of cysteine. All tested fungi showed considerable amount of cysteine released. Maximum cysteine was released by *Chrysosporium anamorph of* In comparison to cysteine, the overall release of cystine was higher in all the fungal isolates throughout the incubation time. Maximum cystine released during shake condition was observed in case of *Geomyces pannorum* (9.95µg/ml), followed by *Trichophyton mentagrophytes* (9.60µg/ml), *Chrysosporium anamorph of Rollandina vriesii* (7.81µg/ml) and *Microsporum fulvum* (6.96µg/ml). *Geomyces pannorum* was the best performer in stationary condition also. It showed almost similar results in both shake and stationary condition. It released (9.68µg/ml), followed by *Microsporum fulvum* (8.74µg/ml), *Trichophyton mentagrophytes* (7.79µg/ml) and least by *chrysosporium anamorph of rollandina vriesii* (6.93µg/ml) on 28th day of incubation. Until 28 days, the value of cystine was tremendously high when compared to cysteine and then leaded to a sharp decline by 35th day of incubation.

Methionine, another sulphur containing amino acid was also detected in the culture filtrate analyzed. Highest amount of *Rollandina vriesii* (5.08 µg/ml), followed by *Geomyces pannorum* (4.75µg/ml), *Trichophyton mentagrophytes* (4.50µg/ml) and least by *Microsporum fulvum* (0.94µg/ml) in shake condition, whereas in stationary condition instead of *Chrysosporium anamorph of Rollandina vriesii*, *Geomyces pannorum* produced maximum cysteine (3.57µg/ml), followed by *Chrysosporium anamorph of rollandina vriesii* (3.13µg/ml), *Microsporum fulvum* (1.59µg/ml) and least by *Trichophyton mentagrophytes* (0.55µg/ml). The values of cysteine released were depicted in a zigzag manner, showing an initial increase with a sharp decline on 21st day of incubation and then showing a gradual increase with further rise in incubation-period.

methionine was extracted from *Geomyces pannnorum* (362.26µg/ml and 228.81µg/ml), followed by *Chrysosporium anamorph of Rollandina vriesii* (328.82µg/ml and 213.42µg/ml), *Trichophyton mentagrophytes* (220.44µg/ml and 200.82µg/ml) and minimum methionine released was observed in *Microsporum fulvum* (185.49µg/ml and 97.11µg/ml) in both shake and stationary condition in 21 days respectively. The rate of methionine release increased upto 28 days and showed a tremendous fall by the end of incubation period in culture filtrates of *Chrysosporium anamorph of Rollandina vriesii* and *Microsporum fulvum*, but with *Geomyces pannorum* and *Trichophyton mentagrophytes*, methionine release increased up to 21 days with a sharp decline from 28 days onward. In stationary condition, *Geomyces pannorum* and *Microsporum fulvum* showed a zigzag pattern with increase up to 14 days, followed by subsequent decline after 21 days and a slight increase during last phase of the incubation, whereas *Chrysosporium*

anamorph of rollandina vriesii showed tremendous increase up to 28 days, with a sharp decline by the end of incubation period and *Trichophyton mentagrophytes* showed increased release of methionine upto 21 days with rapid fall at 28 days. Hence *Geomyces pannorum* could be considered as the best strain among all test fungi, showing maximum release of cystine (9.95µg/ml and 9.68µg/ml) and methionine (362.26µg/ml and 228.81µg/ml) in both shake and stationary condition respectively. But in culture filtrates of *Geomyces pannorum*, cysteine released was higher only in stationary condition (3.57µg/ml) but in shake condition *Chrysosporium anamorph of Rollandina vriesii* was ahead *Geomyces pannorum* in cysteine production.

In the present study the highest rate of degradation of protein and amino acid (cystine and metheonine) was done by *Geomyces pannorum* in both shake and stationary condition. But in culture filtrates of *Geomyces pannorum*, cysteine released was higher only in stationary condition, in shake condition *Chrysosporium anamorph of Rollandina vriesii* was ahead *Geomyces pannorum*, in cysteine production. Sowjanya et al., (2012) reported the amount of protein released from different avian feathers by *Microsporum gypseum*. Najwa (2013) studied the degradation activity of 112 species of keratinophilic fungi on horns, hedgehog quills and hairs. Kumawat et al., (2013) keratinolytic enzyme production by *Trichophyton mentagrophytes* and *Microsporum canis* and the fungal digestion of animal horn, chicken feathers, finger nails, animal hairs and human hairs by keratinophiles such as *Microsporum gypseum, Microsporum canis, Trichophyton mentagrophytes and Trichophyton rubrum*. Mini K. D et al., (2012) assayed maximum keratinase production. Tambekar et al., (2007) reported, release of protein by utilizing hair keratin. *Microsporum gypseum* 500µg/ml, 400µg/ml *Microsporum canis*, 200µg/ml *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton tonsurans* 180µg/ml in *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus* after 60 days of incubation. Kumar and Kushwaha et al., (2015). Estimation of protein release and amino acids. Amount of protein is released by *Chrysosporium tropicum, Penicillium griseofulvum and Aphanoascus terreus*, 432.66µg/ml, 359.33µg/ml and 339.66µg/ml respectively. R. kanchana et al., (2013) complete degradation of chicken feather within a period of 96 h by *Aspergillus* strain that involve hydrolysis of keratin, improvement of the nutritional properties of feathers (and other keratins) used as supplementary feedstuffs. Maruthi et al., (2011). Degradation of feathers and hair was assessed by a extremely potent keratinophilic fungi particularly *Chrysosporium tropicum*. Kumar et al., (2014) the feather degradation and keratinase producing ability. *Acremonium strictum* (74.40Unit/ml & 124.72 Unit/ml in 8 & 12 day incubation severally, whereas *Chrysosporium indicum* 110.10U/ml and *Chrysosporium tropicum* 78.64U/ml was found highest keratinase production. Godheja et al., (2014) study deals with identification of fungi that play a significant role in the degradation of chicken feather and keratin degradation ability of the isolated fungi.

Conclusion

This group of fungi is known to degrade keratin, a form of protein into amino acids by enzymatic action of keratinase. Thus, the biotechnological aspect was to screen out keratinophilic microbes with high degradation capacity, which can be used as a biotechnological tool to obtain products of human interest by utilizing keratin waste. These isolates with exceptional performance further strengthen the concept of microbial enzyme technology. these hydrolytic products can be used as a food supplement on one hand and enzyme keratinase, having industrial importance on the other hand. Keratinophilic moulds have been found useful in the aspect as they help in production of amino acids and other useful enzymes and proteins.

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Incubation Period (In Days)		*Chrysopsorium anamorph of Rollandina vriesii*								*Geomyces pannorum*							
		Shake Condition				Stationary Condition				Shake condition				Stationary Condition			
		Protein	Cyst-eine	cystine	Methe onine	protein	Cyst-eine	cystine	Methe onine	Protein	Cyst-eine	cystine	Methe onine	protein	Cyst-eine	cystine	Methe Onine
7	Net (µg/ml)	0.92	4.06	0.27	127.67	0.42	2.89	0.45	124.12	3.05	1.26	2.15	240.33	1.39	1.25	0.74	195.13
		0.90	4.02	0.25	127.22	0.46	2.85	0.47	122.78	4.06	1.36	2.20	237.64	1.41	1.40	0.76	209.03
		0.95	4.09	0.28	129.91	0.44	3.00	0.44	121.21	3.19	1.49	2.23	242.34	1.46	1.43	0.80	200.08
	Mean±SD T- value	0.92± 0.02 (0.02)	4.06± 0.03 (0.04)	0.26± 0.01 (0.02)	128.22± 1.44 (0.45)	0.44± 0.02 (-0.04)	2.92± 0.07 (0.03)	0.45± 0.01 (-0.02)	122.70± 1.46 (1.34)	3.43± 0.55 (-1.01)	1.37± 0.11 (-0.10)	2.19± 0.04 (-0.05)	242.10± 2.36 (2.69)*	1.42± 0.04 (-0.02)	1.36± 0.10 (-0.15)	0.77± 0.03 (-0.02)	201.41± 7.04 (-13.90)
14	Net (µg/ml)	4.88	5.19	1.59	220.02	1.59	3.55	0.54	179.49	4.71	3.96	5.84	279.03	4.57	2.57	2.43	243.90
		4.87	4.95	1.41	222.49	2.12	2.85	0.60	184.86	4.56	5.10	5.65	267.84	4.68	4.06	2.37	245.24
		4.93	5.09	1.54	225.62	2.05	2.99	0.55	173.23	4.62	5.18	5.55	248.15	4.61	4.09	2.28	243.00
	Mean±SD T- value	4.89± 0.03 (0.01)	5.08± 0.12 (0.24)	1.51± 0.09 (0.18)	222.71± 2.81 (-2.47)	1.92± 0.29 (-0.53)	3.13± 0.37 (0.70)	0.56± 0.03 (-0.06)	179.19± 5.82 (-5.37)	4.63± 0.07 (0.15)	4.75± 0.68 (-1.14)	5.68± 0.15 (0.19)	265.01± 15.63 (11.19)**	4.62 ± 0.06 (-0.11)	3.57± 0.87 (-1.49)	2.36± 0.07 (0.06)	244.05± 1.13 (-1.34)
21	Net (µg/ml)	6.38	0.60	2.12	360.89	3.12	1.11	1.52	214.32	7.26	1.64	7.09	377.03	5.92	1.63	5.22	220.16
		6.11	0.52	2.23	292.19	3.02	1.04	1.85	212.53	7.38	1.58	6.92	387.32	6.16	1.57	5.11	220.83
		6.13	0.48	2.03	333.37	2.15	1.06	1.84	213.42	7.47	1.65	6.96	322.43	6.21	1.58	5.16	245.45
	Mean±SD T- value	6.21± 0.15 (0.27)	0.53± 0.06 (0.08)	2.13± 0.10 (-0.11)	328.82± 34.57 (68.70)**	2.76± 0.53 (0.10)	1.07± 0.04 (0.07)	1.74± 0.19 (-0.33)	213.4± 0.89 (1.79)	7.37± 0.10 (-0.12)	1.62± 0.04 (0.06)	6.99± 0.09 (0.17)	362.26± 34.87 (-10.09)	6.10± 0.15 (-0.24)	1.59± 0.03 (0.06)	5.16± 0.05 (0.11)	228.81± 14.41 (-0.67)
28	Net (µg/ml)	5.83	1.25	7.94	344.85	2.27	0.42	7.06	219.53	7.21	2.22	10.06	247.39	5.25	1.74	9.82	228.14
		5.90	1.42	7.75	359.17	2.03	0.35	6.79	217.74	7.13	2.64	9.89	249.85	5.28	1.89	9.67	237.31
		5.46	1.31	7.75	396.09	2.00	0.48	6.95	243.48	7.08	2.26	9.91	240.90	5.25	1.79	9.55	241.57
	Mean± SD T- value	5.73± 0.24 (-0.07)	1.33± 0.09 (-0.17)	7.81± 0.11 (0.19)	366.70± 26.44 (-14.32)	2.10± 0.15 (0.24)	0.42± 0.06 (0.07)	6.93± 0.14 (0.27)	226.92± 14.37 (1.79)	7.14± 0.07 (0.08)	2.37± 0.23 (-0.42)	9.95± 0.09 (0.17)	246.05± 4.62 (-2.46)	5.26± 0.02 (-0.03)	1.81± 0.08 (-0.15)	9.68± 0.13 (0.15)	235.67± 6.86 (-9.17)
35	Net (µg/ml)	2.52	1.26	1.97	168.89	1.50	0.78	1.50	124.36	2.63	1.21	6.02	244.33	2.56	0.87	2.37	154.82
		2.38	1.54	2.03	183.21	1.51	0.81	1.64	128.61	2.49	1.21	6.15	242.32	2.55	0.86	2.30	157.73
		2.38	1.40	2.07	177.84	1.55	0.84	1.58	138.45	2.61	1.18	6.19	242.54	2.42	0.82	2.27	161.98
	Mean±SD T- value	2.43± 0.08 (0.14	1.40± 0.14 (-0.28)	2 .02± 0 .05 (-0.06)	176.65± 7.23 (-14.32)	1.52± 0.03 (-0.01)	0.81± 0.03 (-0.03)	1.57± 0.07 (-0.14)	130.47± 7.23 (-4.25)	2.57± 0.08 (0.14)	1.20± 0.02 (0.00)	6.12± 0.09 (-0.13)	243.06± 1.10 (2.10)*	2.51± 0.08 (0.01)	0.85± 0.03 (0.01)	2.31± 0.05 (0.07)	158.18± 3.60 (-2.91)

SD = Standard deviation, (T-Values)*-significant, (T-Values)**-highly significant

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Incubation Period (In Days)		FUNGI															
		Microsporum fulvum								*Trichophyton mentagrophytes*							
		Shake Condition				Stationary Condition				Shake Condition				Stationary Condition			
		protein	cyst-eine	cystine	metheonine	protein	cyst-eine	Cystine	Metheonine	protein	cyst-eine	cystine	metheonine	protein	Cyst-eine	cystine	Methe Onine
7	Net (µg/ml)	1.16	0.58	1.30	136.57	1.19	1.07	0.73	140.63	1.03	3.30	1.34	122.55	0.94	0.45	0.37	87.67
		1.18	0.66	1.26	136.12	1.15	1.23	0.79	133.25	0.92	3.45	1.40	123.67	1.07	0.54	0.38	84.76
		1.16	0.71	1.40	139.93	1.24	1.26	0.77	136.16	1.16	3.36	1.29	125.01	0.96	0.56	0.39	89.24
	Mean± SD T- value	1.16± 0.01 (0.00)	0.65± 0.07 (-0.08)	1.32± 0.07 (0.04)	137.5± 2.08 (0.45)	1.19± 0.04 (0.04)	1.19± 0.10 (-0.16)	0.76± 0.03 (-0.06)	136.68± 3.72 (2.91)*	1.04± 0.12 (0.11)	3.37± 0.07 (-0.15)	1.34± 0.05 (-0.06)	123.74± 1.23 (-1.12)	0.99± 0.07 (-1.00)	0.52± 0.06 (-0.09)	0.38± 0.01 (-0.01)	87.22± 2.27 (2.91)*
14	Net (µg/ml)	1.15	0.27	1.40	152.25	2.37	0.87	2.32	152.67	1.30	4.02	1.83	169.98	1.30	0.54	0.21	164.58
		1.15	1.14	1.49	152.92	2.31	2.06	2.30	156.47	1.32	4.73	1.71	171.77	1.38	0.49	0.22	166.37
		1.17	1.42	1.45	157.84	2.34	1.85	2.14	155.58	1.33	4.76	1.70	170.87	1.39	0.61	0.25	165.25
	Mean± SD T- value	1.16± 0.01 (0.00)	0.94± 0.60 (-0.87)	1.45± 0.04 (-0.09)	154.34± 3.05 (-0.67)	2.34± 0.03 (0.06)	1.59± 0.63 (-1.19)	2.25± 0.10 (0.02)	154.91± 1.99 (-3.80)	1.32± 0.01 (-0.02)	4.50± 0.42 (-0.71)	1.75± 0.07 (0.12)	170.87± 0.89 (-1.79)	1.36± 0.05 (-0.08)	0.55± 0.06 (0.05)	0.23± 0.02 (-0.01)	165.40± 0.90 (-1.79)
21	Net (µg/ml)	5.92	0.25	2.32	172.29	2.50	0.05	2.46	92.63	6.19	1.97	6.15	235.58	4.06	1.06	1.40	167.11
		5.65	0.30	2.30	198.92	2.44	0.02	2.41	96.88	5.94	1.90	6.72	203.58	4.01	1.00	1.53	218.35
		5.45	0.37	2.29	185.27	2.57	0.05	2.33	101.81	5.71	1.99	6.32	222.16	3.84	0.87	1.40	217.01
	Mean± SD T- value	5.67± 0.24 (0.27)	0.31± 0.06 (-0.05)	2.30± 0.01 (0.02)	185.49± 13.32 (-26.63)	2.50± 0.06 (0.06)	0.04± 0.02 (0.03)	2.40± 0.06 (0.05)	97.11± 4.59 (-4.25)	5.95± 0.24 (0.25)	1.95± 0.05 (0.07)	6.40± 0.29 (-0.57)	220.44± 16.07 (32.00)**	3.97± 0.11 (0.05)	0.98± 0.10 (0.06)	1.44± 0.07 (-0.13)	200.82± 29.20 (-51.24)
28	Net (µg/ml)	2.87	1.64	7.04	296.66	3.93	1.03	8.83	156.80	4.94	2.18	9.62	174.17	3.97	1.31	8.00	152.69
		2.80	1.67	6.96	292.41	3.84	1.09	8.75	153.45	4.79	2.30	9.53	181.56	3.68	1.25	7.58	154.26
		2.80	1.48	6.87	297.56	3.89	1.13	8.64	159.94	4.68	2.15	9.64	179.77	3.73	1.32	7.79	153.81
	Mean± SD T- value	2.87± 0.04 (0.07)	1.60± 0.10 (-0.03)	6.96± 0.08 (0.08)	295.54± 2.75 (4.25)*	3.89± 0.04 (0.09)	1.08± 0.05 (-0.06)	8.74± 0.09 (0.08)	156.73± 3.25 (3.35)*	4.80± 0.13 (0.15)	2.21± 0.08 (-0.12)	9.60± 0.06 (0.09)	178.50± 3.85 (-7.39)	3.79± 0.15 (0.29)	1.29± 0.04 (0.06)	7.79± 0.21 (0.42)	153.59± 0.81 (-1.57)
35	Net (µg/ml)	3.49	1.47	1.91	174.38	3.62	0.85	2.33	177.51	2.56	1.63	2.17	203.21	1.25	1.19	0.38	136.75
		3.46	1.50	1.88	176.39	3.45	0.98	2.41	181.32	2.26	1.51	2.15	191.57	1.38	1.21	0.45	138.09
		3.60	1.45	1.86	175.94	2.66	0.82	2.47	167.89	2.59	1.41	2.21	198.29	1.11	1.32	0.42	130.48
	Mean± SD T- value	3.51± 0.07 (0.03)	1.47± 0.02 (-0.03)	1.88± 0.02 (0.03)	175.57± 1.05 (-2.01)	3.24± 0.51 (0.17)	0.88± 0.08 (-0.13)	2.40± 0.07 (-0.08)	175.57± 6.92 (-3.81)	2.47± 0.18 (0.30)	1.52± 0.11 (0.12)	2.18± 0.03 (0.02)	197.69± 5.84 (11.64)*	1.25± 0.13 (-0.13)	1.24± 0.07 (-0.02)	0.42± 0.03 (-0.07)	135.11± 4.06 (-1.34)

SD = Standard deviation, (T-Values)*-significant, (T-Values)**-highly significant.

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