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Impact of kinnow peel and nano-limonin on the performance and meat quality of commercial broilers

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ABSTRACT

This study was taken up with the objective to assess the effect of limonin on the performance of commercial broilers and quality of meat. Day old chicks (200) were divided into 8 groups, each group contained 4 replicates of 6 chicks each in equal sex ratio. The iso-nitrogenous and iso-caloric diets were fed for 35 days, i.e. starter, grower and finisher phase. Kinnow peel powder (KPP) and solid lipid nanoparticles (SLN) of kinnow peel powder containing 7.47 mg limonin/g was added in the required quantity of feed to supply 0, 0.5, 1.0 and 1.5 mg limonin/bird/day. The data was analyzed using 2×4 factorial design. The data revealed that the birds fed diet supplemented with SLN consumed more feed in comparison to those fed diet supplemented with KPP, resulting in higher gain in weight, but without affecting feed conversion ratio (FCR). The digestibility of CP was lower and that of CF was higher when diet was supplemented with SLN in comparison to the one supplemented with KPP. As compared to control diet, limonin up to 1% level did not have any adverse effect on the digestibility of nutrients, but it was depressed beyond 1% level of limonin supplementation. The limonin beyond 1% depressed the dressing percentage. It was concluded that nano-formulations @ 1.0 mg/bird/d is an effective carrier of limonins, leading to improved growth, health characteristics in broilers and meat enriched with limonin.

Keywords: Broiler, Kinnow waste, Limonin, Nano-particles, Value-added meat

India's horticulture production has increased by 30% in the last five years and is 2nd highest producer of fruit and vegetables in the world (NHB 2017). At present, only 2.2% of fruits and vegetables produced in India are processed, as compared with 65% in USA, 78% in the Philippines and 23% in China (Krishnakumar 2018). During winter, a citrus fruit variety grown in Punjab and Rajasthan namely kinnow or tangerine (*Citrus reticulata*) is processed into juice by food processing industry. Punjab is the highest producer of mandarin (1.02 million tones/annum; kinnow and orange) in the country and kinnow constitutes 97% of the total citrus produced in the state. After extraction of juice, about 50% of waste (peel, pulp and seeds) is available. Processing of citrus by-products potentially represents a rich source of phenolic compounds, the nutraceuticals. These byproducts are generally discarded as waste causing pollution. But if used judiciously, these wastes are capable of offering a low-cost platform for the production of novel nutritional dietary supplements. Preliminary studies at our centre revealed that it is highly palatable and can serve as an excellent alternate feedstuff for livestock (Wadhwa and Bakshi 2013, Wadhwa *et al*. 2015).

In an era of antibiotic ban, phytochemicals have gained the importance to be used as antibacterial, antiviral, anti-nociceptive, anti-inflammatory, anti-carcinogenic, anti-oxidant and anti-feedant. Guo *et al*. (2003) reported that majority of fruit peels exhibited 2 to 27 fold higher antioxidant activity than the fruit pulp. Devatkal *et al*. (2010) used kinnow rind powder extract as safer alternative to synthetic anti-oxidants in meat products. Rafiq *et al*. (2018) and Celano *et al*. (2019) have identified various phytochemicals and mentioned citrus peel as a source of functional ingredient. Bandyopadhyay *et al*. (2018) extracted polyphenols from food wastes and used for fortification of food with polyphenols for human consumption. Keeping in view the potential of kinnow peel as nutraceutical, a component limonin was extracted to see its impact (as such or its nano form) on broiler performance and retention of limonin in the meat to make it a value-added product for human consumption.

MATERIALS AND METHODS

Limonin extract: Dried kinnow peels are rich in limonoids, flavonoids and carotenoids and these bioactive compounds were extracted by supercritical fluid extraction and entrapped in solid lipid nanoparticles (using stearic acid, tween 20 and glycerol). Both solid lipid nanoparticles (SLN) and kinnow peel powder contained 7.47 mg limonin/g. Pesticides in limonin extract were below detectable limits (got analyzed from PBTI, Mohali, by using Quecher's extraction by GCMS-MS). Limonin extract was free of aflatoxins (got analyzed from Punjab Biotechnology

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Institute (PBTI), Mohali, by using AAC 990.33). Hydrocyanic acid was not detected (got analyzed from PBTI, Mohali, by using DGHS Manual).

Grouping of chicks and dietary treatments: Day-old broiler chicks (Vencobb, n=200) procured from local market were sexed and wing banded at 0 day of age. All chicks were weighed individually and were randomly assigned to 8 dietary groups. Each treatment had 4 replicates having 3 male and 3 female meat type birds. Eight iso-nitrogenous and iso-caloric diets were formulated (ICAR 2013) and fed for 35 days, i.e. starter (0–14 days) grower (15–21 days) and finisher (22–35 days) phase. SLN and KPP were added in the required quantity of feed to supply 0, 0.5, 1.0 and 1.5 mg limonin per bird per day. The diets were analysed for proximate principles, phosphorus (AOAC 2000) and calcium (Talapatra *et al*. 1940) content. The CP level in the diets were 22, 21.5 and 19.5% during respective phases of growth at ME levels of 3,000; 3,050 and 3,100 kcal/kg of feed. Feed and water were offered *ad lib*. Mortality, if any was recorded daily. The birds were reared on deep litter system maintaining standard management practices. Daily known quantity of feed was offered to each replicate. Feed residue left from each replicate was weighed weekly to calculate feed intake and feed conversion ratio.

Growth performance: Body weight of each bird was recorded at weekly interval up to 35 day of age to determine the weekly body weight gain. At the end of feeding trial, four birds (2 male and 2 female) from each treatment were shifted to metabolic cages and metabolic trial was conducted as per the standard procedure.

Blood, meat and carcass evaluation: Four birds (2 male and 2 female) from each treatment were sacrificed by severing jugular vein to assess the carcass and various meat quality parameters including *p*H, cooking yield, water activity, colour profile and sensory evaluation by using standard method. Blood was also collected from jugular vein in vial containing sodium fluoride for blood glucose and in heparinised vials for other parameters. Blood parameters were assayed by using Siemens Autopak kit (Siemens Healthcare Diagnostics Ltd.) on semi-automatic chemistry analyzer (RA 50).

Carcass parameters: The birds were humanely killed after 35 days of feeding. Weight was recorded for carcass, stomach contents and empty body weight. Weights of the breast, thigh, abdominal fat, liver, pancreas, small intestine and cecum were recorded too.

Limonin assay: The breast, leg and liver samples were analyzed for limonin content by HPLC (Breksa and Jr 2007; Abbasis *et al*. 2005) using a VersaMax™ Microplate spectrophotometer (Sunnyvale, CA, USA).

Histopathology: The liver samples were collected in the para-formaldehyde solution and were processed to obtain 5 µ thick paraffin sections. The sections were stained with heamatoxylin and eosin stain and compared with control group to observe any pathological changes in the treatment groups.

Quantification of TNF-alpha: Quantification of TNF-alpha from liver and blood at protein level and *in situ* localized TNF-alpha protein in liver was done by ELISA using Flat-bottomed (Nunc, Maxisorp) plate and Synergy Hi Hybrid Reader (Biolek).

Statistical analysis:The data were analyzed by using 2×4 factorial design using SPSS (version 18, 2010).

RESULTS AND DISCUSSION

The birds fed diet supplemented with SLN consumed more ($P<0.01$) feed in comparison to the one supplemented with KPP, resulting in higher ($P<0.05$) gain in weight, but without affecting feed conversion ratio (Table 1). Bölükbaþý *et al*. (2010) reported improved feed conversion rate, when laying hens were supplemented 1.5 mL/kg bergamot oil (*Citrus bergamia*) in their feed. The daily feed intake was observed to increase ($P<0.01$) with increase in the level of supplementation. The gain in weight was observed to be higher ($P<0.01$) in birds fed diet supplemented with limonin, though no significant differences were observed within the supplemental groups. The digestibility of CP was lower ($P<0.05$) and that of CF was higher ($P<0.05$) when diet was supplemented with SLN in comparison to the one supplemented with KPP. As compared to control diet, limonin up to 1% level did not have any adverse effect on the digestibility of nutrients, but it was depressed ($P<0.01$) beyond 1% level of limonin supplementation. However the digestibility of CP was affected ($P<0.01$) at all levels of limonin as compared to those fed control diet, irrespective of the physical form of limonin (Table 1).

On the contrary, Chaudry *et al*. (2004) have shown that chickens receiving citrus peel had similar feed intake (FI) to the birds in control group. The lower FI of chickens in our study might lead to reduced daily weight gain which could be due to decrease in nutrient digestibility, since soluble fibre alleviates diffusion rate of digestive enzymes into digesta and therefore decreases the nutrient utilization (Annison 1993). The data revealed that neither the level nor the physical form of limonin showed any adverse effect on live weight, dressed weight or weight of internal organs. However supplementation of limonin beyond 1% depressed ($P<0.01$) dressing percentage.

The data revealed that the carcass weight, deboned meat and meat to bone ratio were not affected by supplementation of physical form of limonin, irrespective of the level of supplementation (Table 1). However, as compared to birds fed control diet, the deboned meat was the highest at 1% level of limonin supplementation, but beyond 1% it was depressed, irrespective of the form of limonin. Limonin exerts protective effects on liver toxicity associated with inflammation and tissue injury via attenuation of inflammation and reduction of oxidative stress (Mahmoud *et al*. 2014). On an average the supplementation of limonin in the diets of broilers increased the deboned meat by 14%, irrespective of the type of limonin supplemented, in comparison to the unsupplemented group.

Blood profile: Supplementation of limonin (level or the

Table 1. Effect of limonin on feed intake, utilization of nutrients and performance of broilers

Parameter	Physical form of limonin (PFL)[1]		PSE	Limonin levels, mg/bird/day (LL)[2]				PSE	P value		
	KPP	SLN		Control	0.5	1.0	1.5		PFL	LL	PFL×LL
FI/bird/day	78.9[a]	83.2[b]	0.90	75.7[a]	82.7[b]	82.6[b]	83.3[b]	1.28	0.003	0.001	0.261
Utilization of nutrients, %											
DM	72.4	72.3	0.47	74.6[c]	71.8[ab]	73.0[bc]	70.0[a]	0.68	0.915	0.007	0.120
CP	64.2[b]	61.2[a]	0.84	70.2[c]	59.7[ab]	63.0[b]	57.9[a]	1.19	0.033	<0.001	0.384
EE	90.2	89.8	0.37	91.2[b]	90.8[b]	90.0[b]	87.9[a]	0.53	0.536	0.009	0.160
CF	45.7[a]	50.8[b]	1.35	52.4[bc]	47.4[b]	55.0[c]	38.2[a]	1.91	0.028	0.001	0.001
Performance of broilers											
Gain in wt, g	1372	1422	19.86	1292[a]	1407[b]	1431[b]	1459[b]	28.08	0.085	0.002	0.609
FCR	2.0	2.1	0.01	2.1	2.1	2.0	2.0	0.02	0.129	0.159	0.657
Dressing, %	60.0	59.7	0.31	59.6[ab]	60.7[b]	60.2[b]	58.8[a]	0.43	0.401	0.034	0.040
Liver,%	2.45	2.55	0.11	2.70	2.31	2.42	2.57	0.15	0.515	0.302	0.901
Heart,%	1.70	1.63	0.04	1.69	1.67	1.58	1.71	0.06	0.333	0.073	0.951
Abd fat,%	0.57	0.59	0.01	0.58	0.62	0.54	0.56	0.02	0.310	0.115	0.377
Meat bone ratio											
Deboned meat	604.81	610.81	17.81	548.50[a]	627.38[ab]	650.50[b]	606.88[ab]	24.44	0.777	0.042	0.590
Bone	417.25	420.69	15.30	418.25	420.75	424.12	412.75	30.60	0.875	0.985	0.744
Meat: Bone	1.47	1.46	0.05	1.33	1.51	1.54	1.48	0.07	0.880	0.145	0.559

Irrespective of levels of limonin; [2]Irrespective of physical form of limonin; KPP, Kinnow peel powder; SLN, solid lipid nanoparticles; FI, Feed intake; FCR, Feed conversion ratio; Abd., Abdominal; Figures with different superscripts[a,b,c] in a row differ significantly.

Table 2. Effect of limonin on blood profile broiler chicks, mg/dl

Parameter	Physical form of limonin (PFL)[1]		PSE	Limonin levels, mg/bird/day (LL)[2]				PSE	P value		
	KPP	SLN		Control	0.5	1.0	1.5		PFL	LL	PFL×LL
TP	2.79	2.98	0.11	2.55	2.88	3.09	3.02	0.15	0.246	0.064	0.430
Alb	1.06	1.11	0.03	1.02	1.04	1.14	1.14	0.05	0.342	0.151	0.365
Gb	1.73	1.87	0.08	1.52	1.84	1.95	1.89	0.11	0.243	0.050	0.509
A:G	0.62	0.61	0.01	0.65	0.59	0.61	0.61	0.02	0.757	0.218	0.811
Glucose	230.31	220.19	5.05	227.25	219.12	226.75	227.88	6.90	0.173	0.847	0.331
TG	50.81	61.56	4.85	46.75	58.50	58.75	60.75	7.50	0.134	0.438	0.503
Cholesterol	93.88	99.00	3.88	94.75	103.62	87.50	99.88	5.70	0.364	0.226	0.913
Urea	2.89	2.96	0.24	2.60	2.65	3.11	3.34	0.35	0.857	0.354	0.801
Crt	0.14	0.16	0.01	0.18	0.12	0.15	0.16	0.02	0.317	0.331	0.378
UA	4.34[a]	5.47[b]	0.32	4.20[a]	4.81[a]	6.06[b]	4.55[ab]	0.46	0.021	0.029	0.051
ALT	9.94	11.75	0.85	9.25	10.25	13.25	10.63	1.30	0.154	0.123	0.593
AST	229.25	233.56	10.52	198.00	223.12	256.88	247.62	15.30	0.773	0.300	0.417
ALKP	697.88	607.81	69.14	757.25	638.38	590.88	624.88	101.5	0.374	0.624	0.506
TBIL	0.544	0.563	0.056	0.475	0.413	0.688	0.638	0.082	0.816	0.078	0.129
Calcium	9.58	9.50	0.17	9.10	10.04	9.54	9.49	0.24	0.734	0.096	0.113
Phosphorus	7.04	7.09	0.20	6.35[a]	6.98[ab]	7.30[ab]	7.64[b]	0.30	0.879	0.017	0.865

[1]Irrespective of levels of limonin; [2]Irrespective of physical form of limonin; KPP, Kinnow peel powder; SLN, solid lipid nanoparticles; TP, Total protein; Alb, Albumin; Gb, Globulin; TG, Triglycerides; Crt, Creatinine; UA, Uric acid; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALKP, Alkaline phosphatase; TBIL, Total bilirubin; Figures with different superscripts[a,b,c] in a row differ significantly.

form) to the diet showed no adverse effect on the blood profile, except uric acid, which was observed to be higher (P<0.05) in blood of birds fed limonin as SLN in comparison to the KPP (Table 2), irrespective of the level of supplementation of limonin. The physical form of limonin supplemented showed no difference in the AST levels, but was observed to be higher in the birds fed limonin @ 1%. The levels of P was observed to be higher (P<0.05) in bird fed diet supplemented @ 1.5%.

Abbas *et al.* (2016) reported that *Citrus sinensis* peel

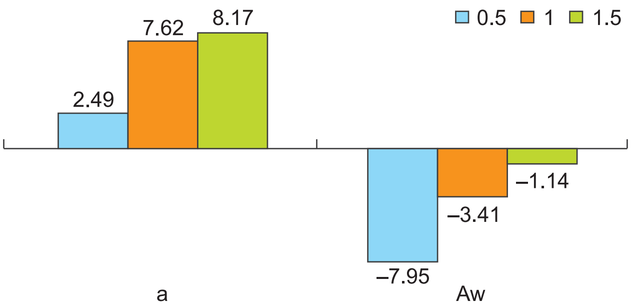

Fig. 1. Per cent change in redness and water activity over control, irrespective of the type of limonin.

did not have any negative effects on selected plasma constituents. On the contrary, Chaudry *et al*. (2004) have shown that blood cholesterol level was significantly reduced with citrus peel inclusion in the diet.

Supplementation with citrus peel (Hong *et al*. 2012) extract (0.2%) appeared not to affect colour parameters of pectoralis major muscle in broilers. The decrease in water activity was observed to be less with increase in level of supplementation of limonin, irrespective of type of limonin in comparison to control group (Fig. 1). The redness of meat increased with increase in the level of supplementation of limonin, irrespective of type of limonin in comparison to control or un-supplemented group (Table 3).

Young *et al*. (2003) and Jang *et al*. (2008) observed no differences in meat water holding capacity, when an oregano supplement (3%) or herb extract mix (0.3–1%), respectively was incorporated in broiler diet. However, combinations of genistein and hesperidin supplementation [5 mg genistein/kg of feed or 20 mg hesperidin/kg of feed or a mixture of genistein and hesperidin (1:4) in doses of 5, 10 or 20 mg/kg of feed] increased water holding capacity values (Kamboh *et al*. 2013). In the present study, on an average supplementation of diet with limonin resulted in increase in redness of meat by 6% and water activity was observed to be reduced by 4% in comparison to the control, irrespective of type of limonin fed.

Limonin content in meat tissues: The results revealed that the bioavailability of limonins in meat was significantly (P<0.01) higher from SLNs as compared to KPP. As compared control group the limonin content in meat was higher (P<0.01) at all levels. However within the levels there was no difference. As compared to control, the limonin content in liver improved (P<0.01) only at 1.0 and 1.5% limonin supplemented groups (Table 4). These observations indicated that nanoformulations is an effective carrier of limonins, leading to improved health characteristics in birds.The bioavailability of citrus limonoids in meat and the recognized biological activity of these compounds in mammalian systems, endorses the reclamation of these compounds for use as nutraceuticals or as healthful fortifiers in functional foods. There was no significant effect of supplementation of limonin in either of the physical form (Table 4) or level of limonin on tumor necrosis factor (TNFα).

Histopathology of Poultry liver: In the birds fed control diet containing no limonin, the histopathology of all samples revealed normal architecture of liver parenchyma,

Table 3. Effect of limonin on colour profile, physico-chemical qualities of broiler meat

Parameter	Physical form of limonin (PFL)[1]		PSE	Limonin levels, mg/bird/day (LL)[2]				PSE	P-value		
	KPP	SLN		Control	0.5	1.0	1.5		PFL	LL	PFL×LL
TP	2.79	2.98	0.11	2.55	2.88	3.09	3.02	0.15	0.246	0.064	0.430
L* value	55.54	55.73	0.12	55.74	56.06	55.36	55.37	0.16	0.296	0.05	0.813
a* value	7.57	7.54	0.08	7.22[a]	7.40[ab]	7.77[b]	7.81[b]	0.11	0.788	0.016	0.243
b* value	17.63	17.62	0.17	17.33	18.02	17.78	17.38	0.24	0.976	0.217	0.808
*p*H	5.84	5.82	0.03	5.86	5.75	5.86	5.84	0.05	0.689	0.370	0.081
Aw	0.86	0.85	0.05	0.88[c]	0.81[a]	0.85[b]	0.87[bc]	0.07	0.344	<0.001	0.018

[1]Irrespective of levels of limonin; [2]Irrespective of physical form of limonin; KPP, Kinnow peel powder; SLN, Solid lipid nanoparticles; L*, Lightness; a*, redness; and b*, yellowness; Aw, Water activity; Figures with different superscripts[a,b,c] in a row differ significantly.

Table 4. Effect of limonin supplementation on meat limonin content, ug/g tissue and tumor necrosis factor (TNF), pg/ml

Parameter	Physical form of limonin (PFL)[1]		PSE	Limonin levels, mg/bird/day (LL)[2]				PSE	P-value		
	KPP	SLN		Control	0.5	1.0	1.5		PFL	LL	PFL×LL
Meat	3.7[a]	25.6[b]	2.36	0.0[a]	18.2[b]	20.5[b]	19.9[b]	3.34	<0.001	0.007	0.006
Liver	25.7	30.6	2.25	0.0[a]	9.4[a]	46.6[b]	56.7[b]	3.18	0.164	<0.001	<0.001
TNFα	99.5	78.0	15.16	96.4	75.4	84.0	99.2	21.38	0.329	0.864	0.785

[1]Irrespective of levels of limonin; [2]Irrespective of physical form of limonin; KPP, Kinnow peel powder; SLN, Solid lipid nanoparticles; Figures with different superscripts[a,b,c] in a row differ significantly.

hepatocytes were normal in appearance, no infiltration and inflammation was seen. Supplementation of diet with KPP 0.5 mg/bird/d resulted in mild inflammation reaction in the form of infiltration of mononuclear inflammatory cells in the parenchyma as well as around the blood vessels, with further increase in level of supplementation to 1 mg/bird/d showed inflammatory reaction in the parenchyma. Many focal areas of inflammation around blood vessels were observed. Supplementation of diet with KPP @1.5 mg/bird/d revealed infiltration of mononuclear inflammatory cells in the parenchyma, mononuclear cell infiltration was seen especially around blood vessels including small blood vessels. There was congestion in the central vein. Whereas, supplementation of diet with SLN @ 0.5 mg/bird/d revealed almost normal histo-architecture with few small areas showing presence of mononuclear inflammatory cells. Increase in level of supplementation of SLN to 1 mg/bird/d revealed very mild inflammation in one case whereas, others were found to be normal in architecture and further increase to 1.5 mg/bird/d showed infiltration of mononuclear inflammatory cells at occasional places in the parenchyma. Mononuclear cell infiltration was seen especially around blood vessels including small blood vessels. There was congestion in the central vein. Limonoid mixtures and purified limonoids have been shown to induce a variety of biological effects in animal and cell culture models (Kim *et al*. 2013, Tundis *et al*. 2014).These effects are indicative of their potential medicinal value and present study is perhaps the first report, indicating value added poultry meat enriched with limonin.

It was concluded that nanoformulations @ 1.0 mg/bird/d is an effective carrier of limonins, leading to improved growth, health characteristics in birds and meat enriched with limonin.

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