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Evaluation of ayurvedic nanoparticle drug *Rasa-sindoora* against gastrointestinal gram-negative bacteria in experimental poultry

Bithika Halder, Samar Sarkar, Samiran Bandapadhyay, Prasanta Kumar Sarkar, Amit Raj Gupta, Subhasish Batabyal and Kaushik Pal

Abstract
Objective: The present study conducted to explore the effects of oral administration of ayurvedic nanoparticle drug *Rasa-sindoora* against gastrointestinal gram-negative bacteria in experimental poultry and haemato-biochemical, pathological alteration due to drug administration.
Materials and Methods: In this case, both *in-vitro* and *in-vivo* experiment were performed. *In-vitro* experiment was carried out by following three methods i.e. agar well diffusion, plate diffusion and disc diffusion method. And *in vivo* experiment was started when the birds were 21 days of age. The birds were divided into three groups i.e. group-C served as control group, group-E treated with Enrofloxacin which act as standard control group and group-RS treated with test drug *Rasa-sindoora*, containing six birds in each group. The experiment (drug trial) was started on the day 22 and continued for next 28 days. On the day 0, 7, 14, 21 and 28 of experiment faecal sample and on day 0, 7, 14 and 28 of experiment blood sample were collected from each bird of three groups to examine the gastrointestinal gram-negative bacterial count and to evaluate the haemato-biochemical parameters i.e. Hb %, SGPT, SGOT, ALP, Urea and Creatinine. After 28 days of experiment, at the age of 50th day, the birds were slaughtered to collect tissue sample from heart, lung, liver and kidney. Illeo-caecal junction tissue was also collected to examine the illeo-caecal coliform count.
Results: *In-vitro* assessment showed no antibacterial efficacy but in *in-vitro* experiment fecal coliform count decreased significantly in RS treated group in comparison to control group. There were no significant ($p<0.01$) alteration in haemoglobin percentage, urea and creatinine level but serum SGPT, SGOT and ALP increased significantly in RS treated group. The illeo-caecal coliform count was decreased significantly in standard control and *Rasa-sindoora* treated groups in both dilutions 1:50 and 1:100 in comparison to normal control group. The result indicate that the test drug *rasa-sindoora* able to decrease the fecal and illeo-caecal coliform count but histopathological findings clearly showed the drug causes chronic toxicity in birds of RS treated group.

Keywords: Ayurvedic nanoparticle drug, *Rasa-sindoora*, poultry, toxicity

Introduction
The poultry gastrointestinal tract harbours a dynamic microbial community consisting of a large number of bacteria across the whole gut length (Yeoman *et al*., 2012) [11]. The intestinal flora of broiler chicken plays an important role for growth performance and health. The gastrointestinal bacteria can be classified as either pathogenic or commensal organisms. Pathogenic bacteria can harm the host by causing localized or systemic infections and intestinal lesions while commensal bacteria can benefit the host by providing nutrients, metabolic facilitation and competitive exclusion. *Escherichia coli* are normal inhabitants of the gastrointestinal tract of poultry of which some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. At present era, drug resistance in *E. coli* has increased worldwide due to continuous use of various class of antimicrobials, mostly through the oral route, with the aim to prevent and treat diseases, enhance growth and productivity in poultry (Page and Gautier, 2012; Landini and Albarellos, 2015) [6, 4] which not only cause direct threat to poultry production but also a major problem to human clinical medicine. To combat with this situation various nanoparticle drugs containing ultra-fined particles generally ranges from 1-100 nm in diameter (Vert *et al*., 2012) [10] produced by nanotechnology are been studying for testing their efficacy (Singh *et al.*, 2008; Kaur *et al*., 2018) [8, 3]. Many nanoparticle based drug shows potential antibacterial activity against various Gram + ve and Gram -ve bacteria including highly methicillin and carbapenem resistant strains and increasing their exposure among animals and human (Nazifi *et al*., 2015; Stebounova *et al*., 2011) [5, 9].

In recent time, nanotechnology is a rapid growing technology plays an important role on various fields of therapeutic applications and capable for solving several problems related to animal health and production (Youssef *et al.*, 2019) [12]. On the other hand, Ayurvedic system of medicine is one of the oldest systems of Indian traditional medicine and most of them are based on herb-metal/mineral formulation commonly known as bhashma having particles diameter of about 10-15nm (Farooq *et al.*, 2019) [1]. Many scientific literatures support their therapeutic efficacy along with metal nanoparticle has been detected in various types of bhashma. So it can be expected that combination of Ayurveda and nanotechnology may provide the solution to solve the antimicrobial resistance issue in poultry production. Few research has reported that mineral rich nanoparticle drug which is Ayurvedic herb-metallic nano medicine have antimicrobial potential assessed by *in vitro* assay (Ruidas *et al.*, 2019; Halder *et al*., 2022) [7, 2].

In this research work a selected ayurvedic nanoparticle drug *Rasa-sindoora* have taken into consideration for studying its effects against gastrointestinal gram-negative bacteria in experimental broiler poultry. This effect was size and dose dependent.

Materials and methods

In-vitro Assay

The *in-vitro* experiment for assessment of antimicrobial sensitivity profile against MDR bacterial strains of *E. coli* was performed by following three methods i.e. agar well diffusion, plate diffusion and disc diffusion in the laboratory of Indian Veterinary Research Institute (IVRI), Kolkata in West Bengal state under the supervision of Dr. Samiran Bandapadhyay, senior scientist of Veterinary Microbiology department.

Agar Well Diffusion Method

Muller-Hinton Agar medium was prepared, sterilized and poured into the sterile petri plates and was allowed to solidify. By using cotton swab Culture of *E. coli* strain was uniformly spread on to the plates containing media. 1 mg of *Rasa-sindoora* was mixed with 1 ml of Solvent (DMSO/Diethyl ether/ Chloroform/ Distilled water) to prepare a suspension. By using gel puncture well was made in the agar media. And the well was filled with the suspension. Each hole contain 200 µl of suspension. The plates were kept carefully in incubator for overnight.

Plate Diffusion Method

250 ml Muller-Hinton Agar media is mixed with different concentration of *Rasa-sindoora* i.e. 2.5 mg, 5 mg and 10 mg. The media was placed into sterilized petri plates and leave it to solidify. 1 microlitre of 0.5 MacFarlane Bacterial culture were placed into that petri plates and kept in incubator for overnight after drying.

Disc Diffusion Method

Muller-Hinton Agar medium was prepared, sterilized and poured into the sterile petri plates and was allowed to solidify. By using cotton swab bacterial culture was uniformly spread on to the plates containing the media. To make suspension 100 mg of *Rasa-sindoora* was dissolved in 250 microliters of Methanol and 500 microliters distilled water. By using Micropipette measured volume of suspension were taken in each disc to make different concentration i.e. 2.5 mg, 5 mg, 10 mg for evaluation of zone of inhibition. After drying the discs were placed on the previously swabbed petri plates and kept in incubator for overnight.

In-vivo Assay

The experiment was conducted at the poultry unit of the 'Ashokenagar Krishi Vigyan Kendra', North 24 Paraganas in West Bengal state on the month of February, 2019 and continued for 7 weeks. At that time, the climatic condition of this area is suitable for poultry farming. Total eighteen (18) day-old broiler chicks were taken for this experiment. The birds were managed on deep litter pens. The chicks were fed broiler starter diet for four weeks and the finisher diet from 5 weeks. At the age of 2 and 4 weeks, the chicks were vaccinated against Gumboro disease and against Newcastle disease at the age of 3 and 5 weeks. At 21 days of age, the birds were divided into three groups i.e. group-C served as control group, group-E treated with Enrofloxacin which act as standard control group and group-RS treated with test drug *Rasa-sindoora* containing six birds in each group. On the day 22, the experiment (Drug trial) was started and continued for next 28 days [Table. 1].

Table 1: Design of the experiment for evaluation of therapeutic potential of herbal compounds in poultry

Group	Number of birds	Used drugs	Dose and route
Group- C	6	No drug	----
Group- E	6	Enrofloxacin	@ 5 mg/kg b. wt., orally once daily for 28 days
Group- RS	6	*Rasa-sindoora*	@ 33.34 mg/kg b. wt., orally once daily for 28 days

*Design of the experiment for evaluation of therapeutic potential of herbal compounds in poultry:

Fecal Sample Examination

Fecal sample was collected on the day 0, 7, 14, 21 and 28 of experiment by using fecal swab from each bird of three groups to examine the gastrointestinal coliform count. In the laboratory one spatula of faecal sample was measured from each sample. Then 1000 microlitre of NSS was added to each sample and unit weight is calculated by dividing NSS with the amount of faecal sample obtained. Finally this unit weight obtained was made up to a volume of 1000 microliters by adding equivalent amount of NSS to it. Then 120 microliters was withdrawn from these and inoculated in MacConkey with CTX agar for bacterial culture [Fig 1.] and the petri plates were kept in incubator for overnight.

Haemato-biochemical Examination

On the day 0, 7, 14 and 28 of experiment blood sample was collected from wing vein with the help of tuberculin syringe in sterile vials under aseptic condition to evaluate the haemato-biochemical parameters i.e. Hb%, SGPT, SGOT, ALP, Urea and Creatinine. The haemoglobin percentage was estimated by using colorimeter and the biochemical tests were performed by using Ebra Chem. 5x model machine (Transition Biomedical Limited) in the biochemistry laboratory, W.B.U.A.F.S., Kolkata (West Bengal). For determination of serum SGPT, SGOT, ALP, Urea and Creatinine level specific reagents and methods were used. The reagent and sample are mixed well and aspirate. Then allow

the reagents to attain 15-30°C before performing the test. To estimate the haemoglobin percentage Drabkin's solution is used.

Histopathological Examination

At the age of 50th day, the birds were slaughtered for tissue sample collection from heart, lung, liver and kidney. Then preserved into 40% formalin in order to perform histopathology, embedded in paraffin, sectioned at 5 µm then stained with haematoxylin and eosin (H&E) for light microscopic examination.

Illeo-caecal junction tissue was also collected to examine the coliform count. A small part of illeo-caecal junction tissue is taken and the weight of tissue is measured. Then the tissue was dissolved in 5 ml of Normal Saline Solution (NSS). The tissue dissolved in NSS is homogenized with a homogenizer. Then the unit weight of tissue was calculated by dividing the weight of tissue with NSS with the tissue weight and the unit weight of tissue was cultured on 1:50 and 1:100 dilution in MacConkey with CTX agar plate for bacterial culture [Fig 2.]. The petri plates were kept for overnight in incubator.

Results and Discussions

Table 2: Result of fecal coliform count

Days Treatments	0 Day	7 Days	14 Days	21 Days	28 Days
Control	16.35±1.46^{b}	72.75±10.25^{b}	114.25±28.26^{b}	3.45±1.25^{b}	81.25±10.23^{b}
Enrofloxacin	12.25±2.35^{b}	17.45±5.26^{c}	19.95±5.62^{c}	46.25±8.95^{a}	16.45±1.65^{c}
RS	19.73±1.36^{b}	36.84±13.56^{c}	122.54±36.34^{a}	8.67±1.62^{b}	32.54±5.12^{c}

Significant at 1% level of significance. Different superscripts (a, b and c) differ significantly at 1% level of significance (p<.01) according to Tukey's Honest Significance Difference Post hoc test

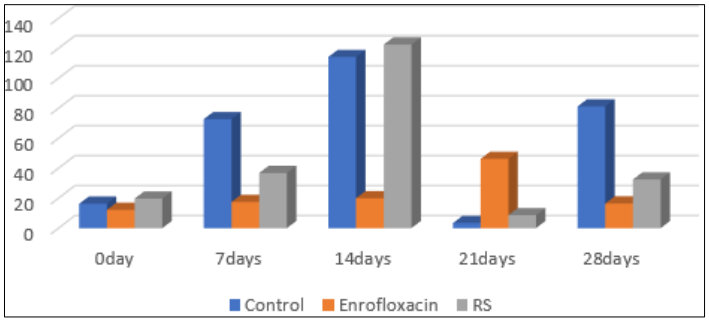

Diagram 1: Fecal coliform count

Fecal coliform count was increased from 0th day to 14th day, then it was decreased in 21st day and again was increased in 28th day in control group and in RS group [Table 2. & Diagram 1.]. In test drug group, highest count observed on 21st day and it decreased on 28th day. On 28th day, the count in RS treated group was less in comparison to control group. All the results are statistically highly significant.

Table 3: Result of Haemoglobin% (gm/dl) Mean ± Standard Error

Days Treatments	0 Days	7 Days	14 Days	28 Days
Control	8.28±.46	7.96±.32	8.45±.36	9.02±.26
Enrofloxacin	8.74±.38	8.68±.21	9.21±.39	9.98±.34
RS	10.56±.27	9.98±.46	10.18.±.35	9.96±.29

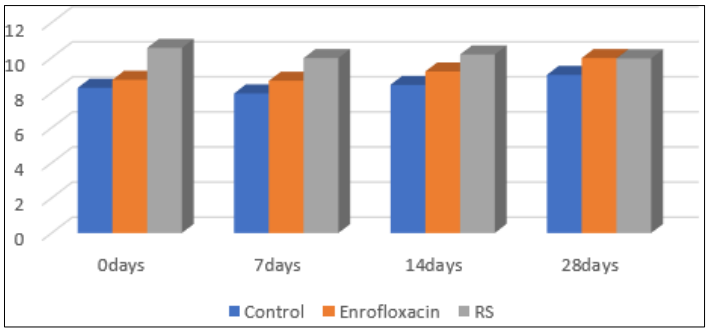

Diagram 2: Haemoglobin % (gm/dL)

Haemoglobin contents in test and control group have been shown in Table 3 & diagram 2. Haemoglobin level alteration was statistically non-significant in all the groups. However, the increases were more in standard control and test RS treated groups in comparison to the normal control group.

Table 4: Result of SGPT/ALT (IU/L) Mean ± Standard Error

Days Treatments	0 Days	7 Days	14 Days	28 Days
Control	21.42±3.38	13.27±.62	19.25±1.25	21.48±1.84
Enrofloxacin	23.54±2.98	13.95±1.95	17.35±1.65	19.62±2.14
RS	26.34±1.87	23.65±5.64	20.87±2.58	35.54±1.36

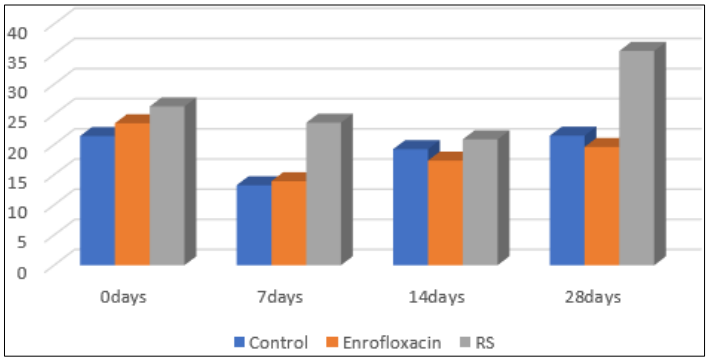

Diagram 3: SGPT/ALT (IU/L)

Table 4. & diagram 3. depicts effect of the control and test drug on serum ALT level. The test drug RS alter serum ALT to significant level. The ALT levels were decreased on 7th and 14th day, again increased on 28th day which clearly indicates hepatic disorder in test birds.

Table 5: Result of SGOT/AST (IU/L) Mean ± Standard Error

Days Treatments	0 Days	7 Days	14 Days	28 Days
Control	186.27±28.25	255.45±27.25	223.42±28.29	248.25±17.65
Enrofloxacin	182.45±10.35	221.25±39.25	192.55±16.45	225.15±14.25
RS	236.54±21.14	258.17±42.53	264.87±34.62	298.68±18.56

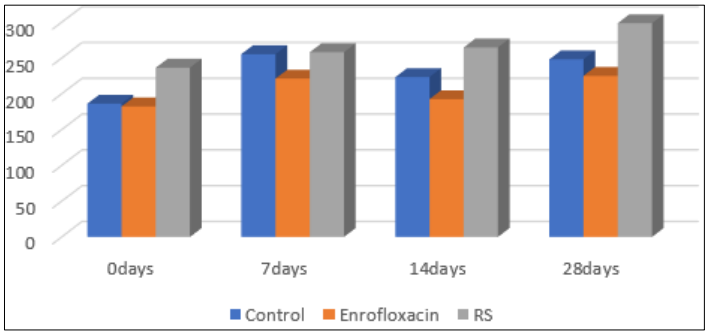

Diagram 4: SGOT/AST (IU/L)

Effects of the control and test drug on serum AST level have been shown in table 5. & diagram 4. Serum AST level was increased significantly in RS treated group may be due to hepatic damage.

Table 6: Result of ALP (IU/L) Mean ± Standard Error

Days Treatments	0 Days	7 Days	14 Days	28 Days
Control	1572.28±70.15	1034.68±124.85	1928.35±178.25	2124.54±62.15
Enrofloxacin	1911.62±62.48	2218.25±168.25	2225.45±165.28	2211.48±4958
RS	2392.85±72.46	2585.25±162.45	3228.34±121.25	4563.34±52.46

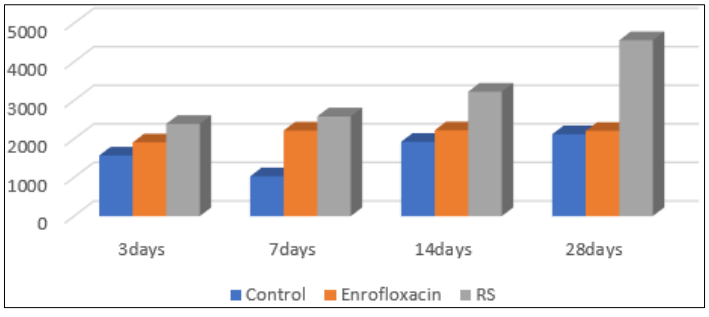

Diagram 5: ALP (IU/L)

Table 6 & diagram 5. shows the results of control and test drug on serum alkaline phosphatase (ALP) level. The test drug alter serum ALP to significant level. Serum ALP level was increased day-by-day in RS treated group indicative for hepatic damage in the experimental birds.

Table 7: Result of UREA (mg/dL) Mean ± Standard Error

Days Treatments	0 Days	7 Days	14 Days	28 Days
Control	9.42±4.05	11.14±7.84	18.26±4.25	12.94±6.27
Enrofloxacin	10.17±3.35	12.25±8.25	12.27±4.95	13.85±7.16
RS	18.34±1.35	25.42±2.01	23.46±2.08	22.19±1.75

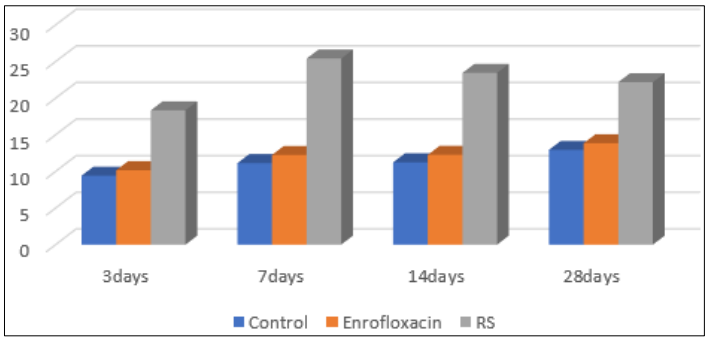

Diagram 6: UREA (mg/dL)

Results on serum urea level were shown in Table 7 & diagram 6. Serum urea level was not altered to significant level by test drug treatment.

Table 8: Result of Creatinine (mg/dL) Mean ± Standard Error

Days Treatments	0 Days	7 Days	14 Days	28 Days
Control	.175±.035	.185±.014	.195±.015	.185±.034
Enrofloxacin	.195±.024	.235±.021	.225±.045	.221±.023
RS	.229±028	.228±.012	.188±.029	.196±.038

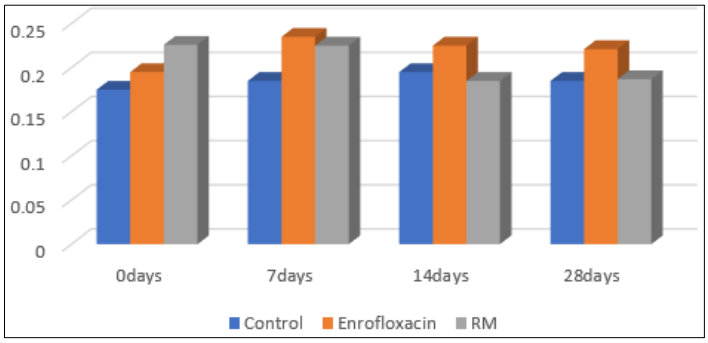

Diagram 7: Creatinine (mg/gL)

Table. 8 & diagram 7. depicts effect of the test drug on serum creatinine level. The test drug didn't alter serum creatinine to significant level.

Table 9: Result of illeo-caecal junction coliform count

Dillution Treatments	1: 50 Dilution	1: 100 Dilution
Control	$38.25\pm.28^{c}$	$24.65\pm.85^{b}$
Enrofloxacin	26.26 ± 1.78^{b}	18.25 ± 14.65^{a}
RS	16.86 ± 16.35^{a}	7.58 ± 16.63^{a}

Significant at 1% level of significance. Different superscripts (a, b and c) differ significantly at 1% level of significance ($p<.01$) according to Tukey's Honest Significance Difference Post hoc test

The illeo-caecal coliform was decreased significantly in standard control and *Rasa-sindoora* treated groups in both the dilutions in comparison to normal control group [Table 9. & diagram 8.]

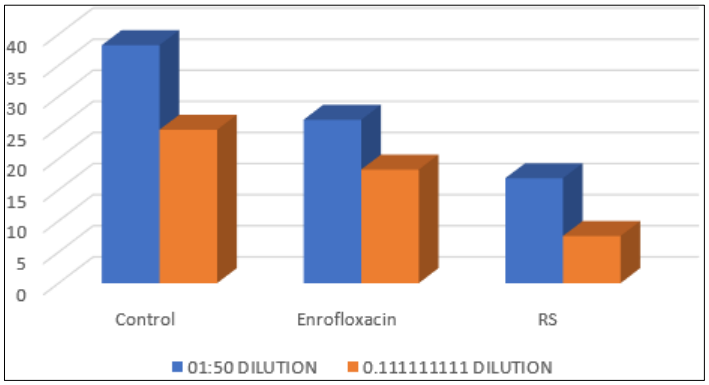

Diagram 8: Illeo-caecal coliform count

Histopathological observation

The test drug, *Rasa-sindoora* (RS) exhibited toxicity in the vital organs which caused degenerative changes in heart [Fig 1.], focal interstitial haemorrhage in lungs [Fig 2.], focal degeneration, central vein congestion, lymphoid accumulation with pseudostratification (Carcinogenic effect) in liver [Fig 3.], zone degeneration, necrotic changes and coagulative changes in kidney [Fig 4.]. *Rasa-sindoora* chemically i.e. mercuric trisulphide (HgS) prepared by following ayurvedic method of purification treatment by melting or by sublimation. These preparation are given for treatment purpose mixing with powder of plant drugs and with specific vehicle according to disease, called as Anupana in parlance of Ayurveda. These are never prescribed as single medicine. The plant drugs and vehicles play important role to increase bioavailability and to reduce toxicity of these preparation in human being.

Before therapeutic application of this compounds in Ayurveda, purification treatment is done. And the drug is prescribed mixing with other plant drugs powder and with specific vehicle according to diseases. Then only this drug is used as therapeutic entity. And at therapeutic dose this drug is found to be safe.

In the present study, *Rasa-sindoora* was given alone without mixing with plant drugs or without any vehicle. Mercury (Hg) compounds are known as carcinogenic agents. Those act as tumor promoting agent and can induce carcinoma. Though before therapeutic application of this compounds, purification treatment is done but as the drug was given without mixing with other plant drugs powder or with specific vehicle, it may be possible cause of toxicity. And another cause may be the drug is not suitable as therapeutic agents in broiler chicken.

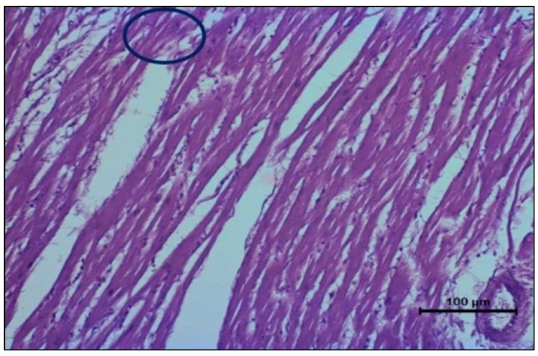

Fig 1: Histopathological findings of heart in RS group of poultry (a) Blue circle represents degenerative changes

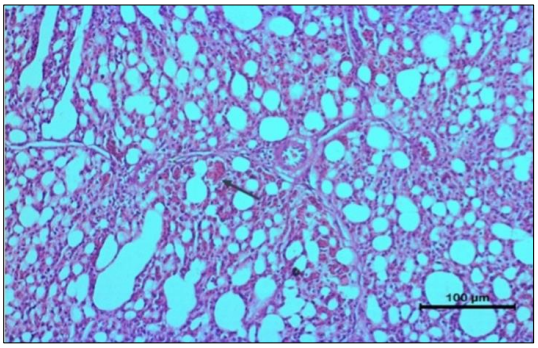

Fig 2: Histopathological findings of lung in RS group of poultry (a) Blue arrow represents focal interstitial hemorrhages

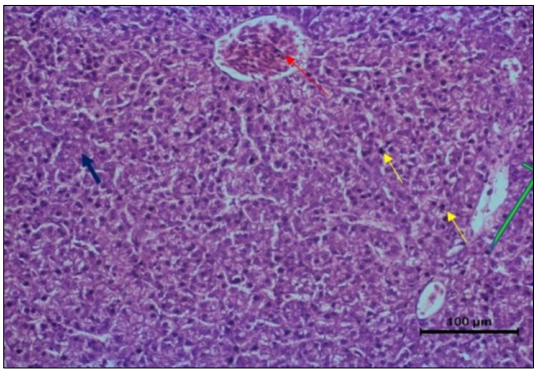

Fig 3: Histopathological findings of liver in RS group of poultry (a) Red arrow represents central vein congestion, b) Blue arrow represents degeneration, c) Yellow lymphoid accumulation, d) Green arrow pseudostratification (Carcinogenic effect)

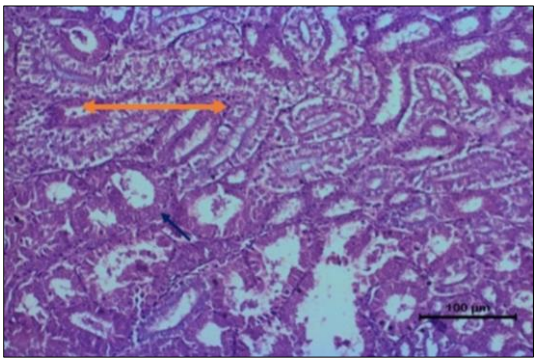

Fig 4: Histopathological findings of kidney in RS group of poultry (a) Orange arrow represents zone degeneration and necrotic change, b) Blue arrow represents coagulative changes

Conclusion

It is concluded that ayurvedic nanoparticle drug *Rasa-sindoora* effective against gastrointestinal gram-negative bacteria and reduces the fecal and illeo-caecal coliform count. But the test drug produces biochemical and pathological alteration in experimental poultry which is correlated to each other and indicates the chronic toxicity in birds of RS treated group.

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