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Supplementation of unleavened flat-bread with lotus root powder exhibits promising antioxidant, anti-inflammatory and analgesic effects: A study involving biochemical and *in vivo* approach

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Abstract: Lotus *(Nelumbo nucifera Gaertn.)* root is known for health-promoting activities due to its high phytochemical content. Therefore, the aim of this study is to increase the amount of health boosting components in commonly eaten product. This was achieved by supplementing lotus root powder (LRP) in refined wheat flour (RWF) and whole wheat flour (WWF) at 0%, 10% and 20% levels to prepare unleavened flat-breads (UFB). This study revealed that addition of LRP increased the ash and fiber content of UFB. Moreover, the antioxidant activity and total flavonoid content also showed increasing trends with the supplementation of LRP in wheat flours. When tested on NMRI mice, UFBs did not show sub-acute toxicity and mortality at 5g/kg dose. Moreover, significant inhibition was observed against carrageenan induced peritonitis, reactive oxygen species formation, acetic acid induced writhes, formalin induced paw edema and hot plate induced jumping in mice at 10% and 20% supplementation levels. Hence, the consumption of LRP incorporated UFB(s) would be more beneficial for consumers to decrease the risk of various diseases.

Keywords: Anti-inflammatory, analgesic, antioxidant activity, toxicity, flavonoid content.

INTRODUCTION

Diet has a remarkable importance on health and well-being of a human. Dietary supplements; specially of natural origin are gaining interests of large population for having wide spectrum of bioactive compounds i.e. flavonoids, alkaloids, tannins, polyphenols, etc. (Perveen *et al.,* 2015). In today's era people are shifting their preferences from synthetic supplements to natural food resources to get tremendous health benefits, these foods are called as functional foods (Abuajah *et al.,* 2015).

Nelumbo nucifera Gaertn. (Lotus) is the part of the *Nelumbonaceae* family. It has been widely grown in Pakistan, Japan, China, India and other countries of Asia. Lotus roots are smooth, 60-140cm long, 0.5-2.5cm in diameter and found in striated brown patches with no enhancing taste or odor (Pal & Dey, 2015). For over 2000 years in Asia, this plant (different parts) has been consumed as vegetable, nutritional/functional food and medicinal herb (Guo, 2009).

Fresh root contains 0.1% fat, 9.7% carbohydrate, 83.8% water, 0.8% fiber, 1.56% reducing sugar, 2.70% crude protein, 0.41% sucrose and 1.1% ash, furthermore, the vitamins (per 100 g) are also present, such as niacin (2.1mg), thiamin (0.22mg), riboflavin (0.6mg) and ascorbic acid (1.5mg) (Panjikkaran *et al.,* 2019). Many minerals; potassium (0.756%), calcium (1.15%), iron (0.053%), zinc (0.0032%), magnesium (0.398%), copper (0.0015%), barium (0.00064%) and sodium (0.10%) are present in lotus root (Mukherjee *et al.,* 2010). Furthermore, antioxidant macro molecular and micro-molecular components have been isolated from lotus root; LB2, gallocatechin and catechin (Jiang *et al.,* 2010). It also possesses medicinal importance, such as for the treatment of dysentery, dyspepsia, diarrhea and piles and also used as cholagogue, nutritive and diuretic agent (Achari *et al.,* 1985). Furthermore, its methanolic extract possesses antipyretic, antimicrobial, antifungal, antibacterial, antidiarrheal, psychopharmacological (ME *et al.,* 2007), anti-diabetic and anti-inflammatory properties (Mukherjee *et al.,* 1997).

Despite the use of *Nelumbo nucifera* as a medicinal food for centuries, its incorporation in unleavened flat-bread and *In Vivo* analysis of this baked product has not been found in literature. Hence, the objective of current study was the fortification of wheat flour with lotus root powder (LRP) to prepare unleavened flat-bread (UFB), as UFB is generally consumed as a staple food in various South Asian countries (Safdar *et al.,* 2009), which makes it the perfect base product for supplementation. The supplementation was done in a most suitable amount as to enhance its taste as well as its quality.

Various tests were performed to identify the credibility of developed functional product. Tests like nutritional composition, antioxidant activity and total flavonoid content, which were followed by *In Vivo* testing on animal to analyze the sub-acute toxicity, anti-inflammatory and analgesic properties and it is expected that LRP supplemented UFB will prevent various chronic diseases.

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MATERIALS AND METHODS

Raw materials

Nelumbo nucifera roots (fresh) were procured from the local market of Karachi, Pakistan. Other ingredients; refined wheat flour (RWF), whole wheat flour (WWF) and salt (National foods, Pakistan), were purchased from the Imtiaz supermarket, Karachi, Pakistan. Sample was identified by the expert.

Chemicals

Catechin (CAS number 154-23-4), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (CAS number 1898-66-4), aluminum chloride (99%) (CAS number 7446-70-0), sodium nitrite (≥99 %) (CAS number 7632-00-0), dexamethasone (CAS Number 50-02-2), k carrageenan (CAS Number 11114-20-8), Evans blue (CAS Number 314-13-6) and diclofenac sodium (CAS Number 15307-79-6) were from Sigma-Aldrich (St. Louis, USA). Formaldehyde (≥37 %) (CAS number 50-00-0) was procured from Carl Roth (Germany) and Paracetamol (CAS number 103-90-2) was obtained from GlaxoSmithKline Ltd. (Pakistan).

Product preparation

Lotus rhizomes were washed, cut into fine slices and oven dried at 70^{o}C for 24 hours. Dried samples were ground and sieved from a 150-mesh size sieve. For UFB preparation, RWF (100g), salt (1gm), water (55±5ml) was used. Same recipe was used for WWF. LRP supplementation was done by replacing RWF or WWF with LRP at different ratios, i.e., 0%, 10% and 20%. The dough was prepared in a dough mixer (Bianchi rapida italia, Italy), sheeted and cooked on heated pan (tawa) on the stove. The prepared UFB(s) was cooled to room temperature (22°C) for 30-45 minutes, dried in an air-drying oven, powdered with the grinder and used in further studies.

Nutritional analysis of UFB(s)

Moisture content by moisture analyzer (51-55, CW brabender, duisbury, NJ, USA), ash content by muffle furnace (KJ-M1400, Kejia furnace co. ltd, China) and protein content by Kjeldahl apparatus (K-350, Buchi) were determined according to AACC Method 44-19.01, AACC Method 08-01.01 and the AACC Method 46-16.01 respectively. AACC Method 30-25.01 and AACC Method 32-10.01 were used to determine the fat content by Soxhlet Apparatus (Thermo fisher scientific, USA) and crude fiber by fiber extractor (Marconi, MA-444, Brazil) respectively (AACC, 2000). The carbohydrate content was measured by subtracting the other components. Atwater general factor system was used in determining the kilocalories.

Antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl)

The antioxidant activity by DPPH was performed in accordance with the method of Zhao *et al.,* (2006) with slight modification. Methanolic extracts (250mg/ml) of each sample (1ml) was taken in a test tube along with 0.5mM DPPH solution (1ml) and kept at dark for about 30 minutes. The absorbance values were measured at 517nm wavelength.

The % scavenging activity is determined as:

$$\% \text{ scavenging activity} = \frac{\text{Abs of control} - \text{Abs o fsample}}{\text{Abs of control}} \times 100$$

Where absorbance of control is the absorbance of all the reagents except the test sample.

Determination of total flavonoid content (TFC)

Extract (250µL) of each sample was added in 5% sodium nitrite (75µL) and left for 6 min. Subsequently, 10% aluminum chloride (150µL) was added along with 1M sodium hydroxide. Distilled water was used to made up the total volume to 2.5ml. The absorbance was taken at 510nm by using a spectrophotometer. For making the standard calibration curve, catechin was used and results were expressed as mg CAE/gm of extract and for UFB samples, mg CAE/100 gm of UFB extract on dry weight (DW) basis (El Atki *et al.,* 2019).

In vivo assays

Animals

Animals were bought from Dow University of Heath Sciences, Ojha Campus, Karachi, Pakistan. The ethical guidelines of OECD were followed for animal handling. The NMRI mice of both sex (23-30g) were used and kept at temperature (22±2ºC) and regulated humidity along with 12h dark/light cycle. Also, the mice were provided with a regular diet and water *ad libitum*.

Ethics statement

For *in vivo* studies, all the experimental methods on mice were conducted with the approval of the Ethical Review Board for Animal Research and Ethics, Dow university of health sciences, Karachi, Pakistan (REF: AR.IRB-018/ DUHS/Approval/2021/028).

Sub-acute toxicity

For determining sub-acute toxicity in mice, highest dose of the compounds was selected. For the continuous period of 7 days, 3/group of mice were given test agents (5g/kg), or pure LRP (5g/kg) or vehicle control saline (0.9%). During this period, animals were kept closely under observation and their behavioral changes and mortality was observed (Siddiqui *et al.,* 2021). After seven days, animals were sacrificed *via* cervical dislocation, their blood was collected and assayed for hematological, liver and kidney function test.

Anti-inflammatory activity

Carrageenan-induced peritonitis

After oral treatment, 0.6% of 10mg/kg Evans blue dye solution was injected intravenously in lateral vein of mice tail and left for 30 minutes. To induce peritonitis, 1% of 0.1mL carrageenan was administered i.p after that. After 4 hours, animals were sacrificed and cold saline (1ml)

was transferred in the peritoneal cavity, followed by its collection and centrifugation at 3000rpm for 5 minutes. The absorbance of supernatant was measured at 620nm (Siddiqui *et al.,* 2016a).

All the treatments were orally given to mice (500mg/kg body weight; n=3 each) (i) 0.9% saline as a vehicle control (ii) UFBs prepared with RWF at 0%, 10% and 20% LRP incorporation (iv) UFBs prepared with WWF at 0%, 10% and 20% LRP incorporation (v) pure LRP (vi) dexamethasone (5mg/kg) as a reference drug (Siddiqui *et al.,* 2016b).

Measurement of reactive oxygen species (ROS) levels

Peritoneal fluid, after centrifugation of control, test reagents (minima and maxima; 0% LRP and 20% LRP in RWF/WWF) and reference drug were collected from carrageenan induced peritonitis. The supernatants were separated and mixed with reagents to measure ROS *(Xian et al.,* 2011). Above stated supernatant (50µL) and same amount of 20µM DCFH-DA (2′, 7′ - dichlorodihydrofluorescein diacetate) were placed in dark in 96 black wells plate at 37ºC for half an hour. Fluorescence microplate reader was used to read absorbance at 485nm 538nm wavelength.

Analgesic activity

Writhing test

Writhing test was performed by following the method of Jo *et al.,* (2021) with slight modification. Reference drug; diclofenac sodium (10, 20, 25mg/kg) (n=3 each) was orally administered. Powdered UFBs; WWF (100, 200, 300mg/kg) (n=3 each) and RWF (100, 200, 300mg/kg) (n=3 each) at 0%, 10% and 20% LRP incorporation and pure LRP (100, 200, 300mg/kg) (n=3 each) was administered against the control (saline 0.9%) (n=3 each). Acetic acid (0.8 % *i.p*) was used to induce the writhes in mice, which is the result of irritation in serous membrane. For 30 minutes, number of writhes were manually counted in animals (treated and control), % inhibition was then calculated.

$$\% \text{ Inhibition} = \frac{\text{Writhes count in control} - \text{Writhes count in treated}}{\text{Writhes count in control}} \times 100$$

Formalin test

Before 30 minutes of the formalin injection (1%, 20µL) on dorsal surface of mice paw, test agents; powdered UFB; WWF (100, 200, 300mg/kg) (n=3 each) and RWF (100, 200, 300mg/kg) (n=3 each) at 0%, 10% and 20% LRP incorporation and pure LRP (100, 200, 300mg/kg) (n=3 each) were administered against the control (saline 0.9%) (n=3 each). Diclofenac sodium (5, 10, 15mg/kg) (n=3 each) was used as a reference drug. Formalin (1%; 20µL) was administered subcutaneously with a 1ml, 27-guage syringe into dorsal surface of right hind paw, which resulted in licking, shaking or nibbling of paw and the time period spent on these behavioral activities was measured. Formalin induced response in animals was categorized into two phases: initial phase and late phase and % inhibition was calculated (Hassanpour *et al.,* 2020).

$$\% \text{ Inhibition} = \frac{\text{Control paw licking (sec)} - \text{Treated paw licking (sec)}}{\text{Control paw licking (sec)}} \times 100$$

Hot plate test

After administering the different doses of test agents; powdered UFB(s); WWF (400, 500, 600mg/kg) (n=3 each), RWF (400, 500, 600mg/kg) (n=3 each), at 0%, 10% and 20% LRP incorporation and pure LRP (400, 500, 600mg/kg) (n=3 each), was administered against the control (saline 0.9%), the temperature of hot plate was set at (50±0.05°C). Paracetamol (300, 400, 500mg/kg) (n=3 each) was selected as a reference drug. Animals were placed on the hot plate, which showed different behaviors in it; which are paw licking and jumping. These behaviors were measured at 30, 60 and 90 minutes along with 30 seconds of cut off time in order to avoid paw damage and identified as latency time (seconds) (Haider *et al.,* 2022).

$$\% \text{ Pain protection} = \frac{\text{Latency (treated)} - \text{Latency (control)}}{\text{Latency (control)}} \times 100$$

STATISTICAL ANALYSIS

All data were analyzed through IBM SPSS Statistics 23. Least significant difference (LSD) test was used to observe the significance level at $P<0.05$, $P<0.01$ and $P<0.005$ in *In Vivo* parameters. Duncan's test was used to observe the 5 % level of significance ($P\leq0.05$) in various parameters. In animals, n=3 was used for each dose, whereas all tests were conducted in triplicate. Therefore, n=9 was used for each dose.

RESULTS

Nutritional analysis of UFB(s)

Nutritional analysis of UFB(s) prepared with LRP-RWF blends is showed in table 1. The supplementation of LRP resulted in an increase in moisture by 3.36%-4.41%, fat by 0.33%-0.68%, ash by 0.95%-1.96% and fiber content by 0.81%-2.82% in UFB(s). However, fat at 0% and 10% incorporation level were insignificantly different ($P\geq0.05$) from each other. Furthermore, protein (10.31%-9.15%), carbohydrate (85.42%-81.25%) and energy (Kcal) (380.9-367.7) significantly decreased ($P\leq0.05$) with the addition of LRP.

In UFB(s) prepared from LRP supplementation in WWF (table 1), the increase in LRP increased the moisture content by 4.56%-5.24%. As LRP is rich in ash (2.46%) and fiber (8.85%), hence its addition proved to increase the ash content (1.64%-2.26%) and fiber content (2.58%-3.82%) of UFB(s). However, its addition decreased the protein by 11.19%-10.19%, fat by 1.84%-1.68%, carbohydrate by 78.19%-76.81% and energy (Kcal) by 374.1-363.2 in UFB(s) prepared with WWF.

Antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl)

Fig. 1a. describes the antioxidant activity of 250mg/ml extracts of UFB (s) made of different ratios of LRP in RWF, which significantly increased ($P \leq 0.05$) with LRP addition and ranged from 34.46-76.89%. Extracts of LRP supplemented UFB(s) made from WWF showed similar pattern as that of RWF, i.e., significantly increased ($P \leq 0.05$) with LRP addition and ranged from 66.37-83.77% (fig. 1a). The antioxidant activity of pure LRP was found to be 86.48%.

Determination of total flavonoid content (TFC)

Fig. 1b. describes the TFC of 250mg/ml extracts of UFB(s) prepared with LRP-RWF ratios, which was increased by the addition of LRP in UFB(s) and ranged from 7.87 to 13.219mg CE/100g dw. In UFB(s) prepared with LRP-WWF ratios, similar increasing pattern in TFC was observed (18.07 to 24.64mg CE/100g dw), with an exception to 10% LRP incorporation level (18.94 CE/100g dw), which showed an insignificant difference ($P \geq 0.05$) with 0% LRP substitution (18.07 CE/100g dw). The highest TFC content was found in pure LRP (31.275 mg CE/100g dw).

Sub-Acute toxicity in mice

The oral administration of UFB(s) prepared with different ratios of LRP (5g/kg) did not demonstrate any toxicological effects or mortality. Furthermore, no behavioral changes (restlessness, agitation, convulsions, dullness, tremors and piloerection) were observed. Biochemical parameters of liver and kidney of all test agents (5g/kg) showed non-significant or similar results as compared to control (table 2). Hematological parameters such as: Hb, RBC, HCT, MCV, MCH, MCHC, WBC and platelet count of all the test agents (UFBs prepared with addition of LRP in RWF and WWF) showed significantly similar results to that of control mice.

Anti-inflammatory activity

Carrageenan-induced peritonitis (vascular permeability)

Upon administration of saline in control animals, the concentration of Evans blue in the peritoneal cavity was found to be 10.1±0.4µg/mL (fig. 2a). The presence of reference drug i.e., dexamethasone (5mg/kg) significantly reduced the Evans blue leakage by 51%. The test agents (500mg/kg body weight); 0% LRP in RWF as well as WWF failed to reduce the Evans blue leakage. However, 10% LRP in RWF, 20% LRP in RWF, 10% LRP in WWF and 20% LRP in WWF significantly reduced ($P<0.005$) the Evans blue leakage by 22%, 42%, 40% and 54% respectively.

Measurement of reactive oxygen species (ROS) levels

The basal levels (control) of ROS in peritoneal fluid were 4813±130 RFU, which was significantly increased (19797±244) by the administration of carrageenan. Reference drug; nordihydroguaiaretic acid (NDGA) significantly reduced ($P<0.005$) the ROS level by 43% (fig. 2b). Test agent; 0% LRP failed to reduce the ROS levels, showing non-significant ($P \geq 0.05$) results. However, at 20% LRP incorporation significant decrease ($P<0.005$) in ROS levels were observed by 21% in RWF and 31% in WWF.

Analgesic activity

Writhing test

The control animals showed 48.8±0.58 writhes. Reference drug; diclofenac sodium (10-25mg/kg) showed significant reduction ($P<0.005$) in abdominal writhes by 25.45%-57.5% against control (table 3). Significant dose dependent inhibitions ($P \leq 0.05$) in LRP: RWF flat-breads were observed at 10% LRP (100-300mg/kg) by 12.81%-25.45% and at 20% LRP (100-300mg/kg) by 18.9%-40.9%. Similar dose dependent inhibitions were obtained in flat-breads prepared by LRP: WWF at 10% LRP (100-300mg/kg) by 15.37%-39.54% and at 20% LRP (100-300mg/kg) by 20.22%-42.72%.

Formalin test

Diclofenac sodium (5-15mg/kg) presented 14-51% and 26-66% attenuation in paw licking in early phase and late phase respectively. However, in control animals, formalin induced paw licking time in early phase was 58.66±3.31 sec, while in late phase was 112±3.28 sec. In early phase, dose dependent reduction ($P \leq 0.05$) in LRP: RWF flat-breads were observed at 10% LRP (200-300mg/kg) by 19.69% and 25.39% and at 20% LRP (100-300mg/kg) by 19.32%-35.79%. During late phase, dose dependent reduction in paw licking was observed at 10% LRP (100-300mg/kg) by 10.02%-32.21% and 20% LRP (100-300mg/kg) by 21.52%-39.58% (table 4).

Similar pattern was observed in LRP: WWF flat-breads. In early phase, at 10% LRP (200-300mg/kg), the reduction in paw licking was 23.86% and 30.11% and at 20% LRP (100-300mg/kg) the % reduction was 26.89%-42.23%. During late phase, dose dependent reduction at 10% LRP (100-300mg/kg) was 13.09%-38.29% and at 20% LRP (100-300mg/kg) was 30.05%-46.92% (table 4).

LRP incorporation (20%) in RWF in late phase and 20% LRP incorporation in WWF in both early phase and late phase showed higher paw licking reduction at 300mg/kg than that of pure LRP (33.53%).

Hot plate test

Control animals showed the latency time of 14-16 sec, measured from 30-90 minutes. In paracetamol (500mg/kg), maximum pain protection was observed at 500mg/kg, i.e. 62.9%, 55.4% and 37.4% at 30, 60 and 90minutes respectively. In LRP: RWF flat-breads, 600mg/kg of 90 minutes showed significant difference ($P<0.05$) from control with % protection of 4.65%. At

10% LRP incorporated UFB(s), no significant pain protection was observed at 400mg/kg on 60th minute. At 30th and 90th minute, significant analgesic effect was observed. At 20% LRP incorporation, significant increase in latency time in dose dependent manner was observed from 400-500mg/kg. Furthermore, highest % protection of test agents was observed on 60th minute at 600mg/kg, i.e. 34.3%. In UFB(s) prepared by adding different ratios of LRP in WWF, test agent; 0% LRP showed % protection of 9.98% at 600mg/kg at 30th minute. At 10% LRP incorporation, 400mg/kg at 60th minute, failed to increase the latency time. At 20% LRP incorporation, highest % protection was observed on 60th minute at 600mg/kg, i.e. 40.14% (table 5).

DISCUSSION

The medicinal importance of lotus rhizome has been known for centuries. It possesses antidiarrheal activity, hypoglycemic activity, antioxidant activity, anti-inflammatory and anti-pyretic properties (Pal & Dey, 2015; Mukherjee *et al.,* 1997). However, its effect on anti-inflammatory and analgesic properties in baked products has not been found in literature. In present study, anti-inflammatory and analgesic properties of the UFB(s) prepared by the addition of LRP has been evaluated.

The nutritional analysis showed that the supplementation of LRP in UFB(s) resulted in an increase in moisture content, probably because of the high water holding capacity of LRP (Bureau *et al.,* 2009). Since, LRP is rich in ash (2.46%) and fiber (8.85%), hence its addition proved to increase the ash and fiber content of UFB(s), this entails the increase in mineral intake. Furthermore, its addition decreased the protein, carbohydrate and energy (Kcal) of UFB(s) prepared with RWF and WWF, as low caloric value would be beneficial for the consumer who requires a restricted calorie intake. With increase in %LRP, fat content of the product decreased in RWF and increased in WWF, as WWF contains germ, which is rich in fat. In contrast to this, Okorie *et al.,* (2002) reported low fat breads made from potato, wheat and cocoyam, ranging from 1.1-1.6%. Similarly, Ahmad *et al.,* (2016) showed the decreased fat and protein content and increased fiber and ash content of cookies with the addition of carrot pomace powder in wheat flour.

The DPPH assay proves to be effective for identifying antioxidant compounds in plant extracts (Gabriel *et al.,* 2022). The obtained results showed that antioxidant activity significantly increased ($P \leq 0.05$) with LRP addition in UFB(s) prepared from RWF and WWF, might be due to the high antioxidant activity of LRP. Comparable results were obtained in some researches, in which addition of LRP increased the antioxidant activity of bread sticks (Thanushree *et al.,* 2017) and antioxidant activity of Chapatti(s) was increased by the incorporation of barley flour in wheat flour (Sharma & Gujral, 2014), Cloning *et al.* (2012) explained the production of brown pigment (specially melanoidins) during baking, which shows a high antioxidant activity in UFB(s).

Flavonoids are known for their contribution of defensive mechanisms in human physiological system (Yadav & Gupta, 2015). The addition of LRP showed the increasing trend in UFB(s) of both RWF and WWF, indicating the higher TFC content of LRP, as it is previously reported that lotus rhizome is rich in bioactive compounds (Panjikkaran *et al.,* 2019) and one of the major compounds; quercetin has been reported to have higher antioxidant activity at high temperatures (Rad *et al.,* 2012). Comparable results were obtained by (Shafi *et al.,* 2016), in which the incorporation of water chestnut flour in wheat flour increased the TFC of cookies. WWF-LRP UFB(s) showed higher TFC than that of RWF-LRP UFB(s), due to the presence of high flavonoid content of wheat bran (3000-4300µg/g) (Fardet, 2010), which is eliminated from RWF, thereby indicating its lower TFC.

The safety of plant based functional foods is of concern, as it may have some toxic effects (Alcorta *et al.,* 2021). The oral administration of UFB(s) prepared with different ratios of LRP (5g/kg) showed no toxicological effects, behavioral changes and mortality. Hence, proved that all the test compounds are non-toxic. Previously reported results showed LD50 values of extracts of *Nymphae lotus L.* rhizome above 5000mg/kg (Murtala *et al.,* 2019), corn silk extract (2000mg/kg) (Ha *et al.,* 2018) and triple fermented barley extracts up to 2000mg/kg dose showed no mortality and abnormal behavior in rats in the 14-day treatment test (Lim *et al.,* 2017).

The peritonitis induced by carrageenan activates the release of histamine, bradykinin, serotonin, leukotrienes, PGs and TNF-α (Smith *et al.,* 1998). The obtained results evaluated that lotus rhizome shows good anti-inflammatory properties, as also reported by Mukherjee *et al.,* (1997). WWF-LRP blends showed higher reduction Evans blue leakage than RWF-LRP blends might be due to the presence of ferulic acid in the bran layer of wheat kernel (Poudel & Bhatta, 2017), which prevents the formation of carcinogens by blocking its reaction with cellular macromolecules (Slavin *et al.,* 2000); thereby showing the anti-inflammatory properties. Similarly, extracts of *Apocynaceae* fruits significantly inhibited the production of cytokines IL-1β, IL-6, IL-12 and TNF-α in carrageen induced peritonitis (Torres-Rêgo *et al.,* 2016) and methanolic extract of *Kigelia pinnata* DC fruit inhibited the migration of peritoneal fluid at the rate of 40.5, 63.1 and 78.9% respectively (Carey *et al.,* 2008).

ROS at prolonged production cause the cell death (Griffith *et al.,* 2009), but they are also responsible for cell growth and functioning at physiological concentrations (Dröge, 2002). The results evaluated the decrease in ROS levels at higher percentages of LRP in

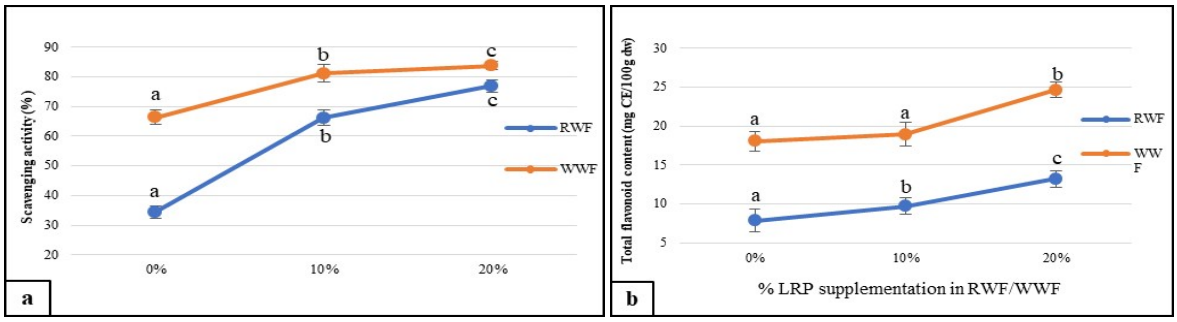

Fig. 1: Bio chemical assays. (a): Scavenging activity (%) of UFB(s) prepared with LRP-RWF and LRP-WWF ratios. However, the % scavenging activity of pure LRP is 86.48%. (b): TFC (mg CE /100g dw) of UFB(s) prepared with LRP-RWF and LRP-WWF ratios. However, TFC of pure LRP is 31.275 mg CE/100g dw.
Each value is expressed as mean ± SD of sample size (n=3). Superscripts written in different alphabets indicate statistically significant difference at $P \leq 0.05$ while the same alphabets indicate statistically insignificant difference at $P \geq 0.05$ among values.

Table 1: Effect of Lotus Root Powder (LRP) supplementation on nutritional properties of unleavened flat-bread (UFB) prepared with refined wheat flour (RWF) and whole wheat flour (WWF).

LRP in flour	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Crude Fiber (%)	Carbohydrate (%)	Energy (Kcal)/100g
Pure LRP	6.91 ± 0.13	7.84 ± 0.12	0.86 ± 0.11	2.46 ± 0.18	8.85 ± 0.17	61.32 ± 0.19	284.4
Refined Wheat Flour							
0% LRP	3.36 ± 0.26^{a}	10.31 ± 0.49^{b}	0.33 ± 0.15^{a}	0.95 ± 0.25^{a}	0.81 ± 0.35^{a}	85.42 ± 0.13^{c}	380.9
10% LRP	3.52 ± 0.25^{b}	10.31 ± 0.94^{b}	0.35 ± 0.83^{a}	1.32 ± 0.14^{b}	1.63 ± 0.24^{b}	84.15 ± 0.16^{b}	378.81
20% LRP	4.14 ± 0.36^{c}	9.15 ± 0.83^{a}	0.68 ± 0.15^{b}	1.96 ± 0.12^{c}	2.82 ± 0.12^{c}	81.25 ± 0.21^{a}	367.7
Whole Wheat Flour							
0% LRP	4.56 ± 0.123^{a}	11.19 ± 0.107^{c}	1.84 ± 0.125^{c}	1.64 ± 0.082^{a}	2.58 ± 0.108^{a}	78.19 ± 0.116^{c}	374.1
10% LRP	4.94 ± 0.169^{b}	10.88 ± 0.145^{b}	1.73 ± 0.108^{b}	1.82 ± 0.146^{b}	2.98 ± 0.167^{b}	77.65 ± 0.135^{b}	369.7
20% LRP	5.24 ± 0.136^{c}	10.19 ± 0.186^{a}	1.68 ± 0.196^{a}	2.26 ± 0.037^{c}	3.82 ± 0.076^{c}	76.81 ± 0.105^{a}	363.2

*Each value is expressed as mean ± SD of sample size (n = 3). Superscripts written in different alphabets within a column indicate statistically significant difference at $P \leq 0.05$ while the same alphabets within a column indicate statistically insignificant difference at $P \geq 0.05$ among values.

Table 2: Effect of lotus root powder (LRP) incorporation in unleavened flat-bread (UFB) prepared with (a) refined wheat flour (RWF) and (b) whole wheat flour (WWF) on liver, kidney and hematological parameters in mice.

Treatments	BIOCHEMICAL PARAMETERS						HEMATOLOGICAL PARAMETERS							
	Bilirubin (mg/dL)		ALT (U/L)	ALP (U/L)	γGT (U/L)	Creatinine (mg/dL)	Hb (g/dL)	RBC (10^6/µL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	WBC (10^9/L)	Platelet count (10^9/L)
	Total	Direct												
Control (saline)	0.76± 0.09	0.09± 0.05	71± 4.2	65± 3.6	7.0± 2.3	0.19±0.7	13.7± 2.1	9.0±1.9	49.5± 1.9	57.2± 4.1	16.3± 3.1	29.4± 2.8	6.7± 2.3	1557±8.3
LRP (Pure)	0.75± 0.05	0.09± 0.02	72 ± 4.2	63± 5.1	7.0± 1.5	0.19±0.7	13.8± 1.5	9.2±1.4	49.6± 2.0	57.8± 3.4	16.5± 2.8	29.5± 2.3	6.5± 2.4	1556±8.5
Refined Wheat Flour														
0% LRP	0.75± 0.02	0.09± 0.04	70± 5.1	67± 6.5	7.1± 1.9	0.17±0.5	13.3± 1.8	9.0±1.9	49.7± 1.7	57.0± 4.0	16.8± 2.7	29.7± 1.7	6.2± 2.6	1555±8.5
20% LRP	0.74± 0.03	0.08± 0.03	72± 3.8	64 ± 5.3	7.3± 1.7	0.16±0.2	13.9± 2.0	9.4±1.4	49.6± 1.2	57.5± 3.2	16.4± 3.1	29.3± 2.4	6.3± 2.3	1556±8.3
Whole Wheat Flour														
0% LRP	0.74± 0.05	0.08± 0.05	72± 7.6	67± 3.3	7.1± 2.9	0.18±0.9	13.4± 1.9	9.1±1.8	49.1± 2.0	57.6± 3.9	16.5± 2.9	29.1± 2.2	6.5± 2.5	1559±8.1
20% LRP	0.73 ± 0.08	0.07± 0.04	72± 4.0	66± 4.2	7.0± 2.2	0.18±0.4	13.5± 1.9	9.3± 1.7	49.8± 2.3	57.3± 3.5	16.7± 2.8	29.5± 1.9	6.4± 2.7	1557±8.2

*Each value is expressed as mean ± SEM of sample size (n=3) of three independent experiments. All test agents were taken at the dose of 5g/kg
Where, ALT = Alanine aminotransferase, ALP = alkaline phosphatase and γGT = gamma glutamyl transferase, Hb = Hemoglobin, RBC = Red blood cell count, HCT = Hematocrit, MCV = Mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, WBC = white blood cells
All the values of treated groups were similar to the control values and hence the non-significant (ns) is not labeled in the table.

Table 3: Effect of Lotus Root Powder (LRP) supplementation in unleavened flat-bread (UFB) prepared with refined wheat flour (RWF) and whole wheat flour (WWF) on acetic acid-induced writhes in mice.

TREATMENTS	DOSE (mg/kg)	NO. OF WRITHES	% INHIBITION
Pure LRP	100	$36.7 \pm 0.77^{***c}$	24.77 ± 1.38
	200	$23.7 \pm 0.87^{***b}$	51.36 ± 1.63
	300	$18.2 \pm 1.09^{***a}$	62.7 ± 2.37
Refined Wheat Flour UFB(s)			
0% LRP	100	$46.7 \pm 1.28^{ns, ab}$	4.32 ± 3.57
	200	$46.2 \pm 1.06^{ns, ab}$	5.45 ± 2.40
	300	$44.5 \pm 1.04^{***a}$	8.86 ± 2.76
10% LRP	100	$42.6 \pm 1.06^{***b}$	12.81 ± 2.19
	200	$39.2 \pm 1.26^{***a}$	19.77 ± 3.20
	300	$36.4 \pm 1.40^{***a}$	25.45 ± 3.52
20% LRP	100	$39.6 \pm 0.84^{***c}$	18.90 ± 1.29
	200	$34.5 \pm 1.62^{***b}$	29.31 ± 4.42
	300	$28.8 \pm 1.66^{***a}$	40.90 ± 4.95
Whole Wheat Flour UFB(s)			
0% LRP	100	$45.4 \pm 1.43^{*a}$	7.04 ± 4.54
	200	$44.6 \pm 1.09^{*a}$	8.63 ± 2.83
	300	$44.5 \pm 1.04^{***a}$	8.86 ± 2.76
10% LRP	100	$41.4 \pm 0.65^{***c}$	15.37 ± 1.04
	200	$36.2 \pm 0.68^{***b}$	25.91 ± 1.49
	300	$29.5 \pm 0.55^{***a}$	39.54 ± 1.49
20% LRP	100	$39 \pm 0.42^{***c}$	20.22 ± 0.99
	200	$35.1 \pm 1.08^{***b}$	28.18 ± 1.59
	300	$28 \pm 0.83^{***a}$	42.72 ± 1.41
Reference drug			
Diclofenac sodium	10	$36.4 \pm 1.04^{***c}$	25.45 ± 0.99
	20	$28.1 \pm 0.81^{***b}$	42.5 ± 1.20
	25	$20.7 \pm 1.40^{***a}$	57.5 ± 2.40
Control		48.88 ± 0.58	

*Each value is expressed as mean ± SEM of sample size (n=3). Superscripts written in different alphabets and numerals within a column indicate statistically significant difference at $P \leq 0.05$ while the same alphabets and numerals within a column indicate statistically insignificant difference at $P \geq 0.05$ among doses of a single group and among highest dose of each group respectively.

The no. of writhes with respect to control (48.88±0.58) represented by asterisks indicating significant difference at ($P<0.05$, **$P<0.01$ and ***$P<0.005$) and non-significant values are indicated by ns.

Table 4: Effect of Lotus Root Powder (LRP) supplementation in unleavened flat-bread (UFB) prepared with refined wheat flour (RWF) and whole wheat flour (WWF) on formalin induced paw licking in mice.

Treatments	Dose (mg/kg)	Early Phase		Late Phase	
		Licking Time (sec)	Reduction in Licking Time (%)	Licking Time (sec)	Reduction in Licking Time (%)
Pure LRP	100	56.4 ± 1.02^{b}	3.78 ± 0.94	105.8 ± 3.22^{c}	5.45 ± 4.79
	200	$51.2 \pm 2.49^{**b}$	12.68 ± 7.37	$96.4 \pm 2.09^{***b}$	13.88 ± 1.30
	300	$42 \pm 1.22^{***a}$	28.4 ± 1.99	$74.4 \pm 1.29^{***a}$	33.53 ± 1.14
Refined Wheat Flour					
0% LRP	100	58.5 ± 2.28^{a}	0.19 ± 4.83	108.8 ± 3.56^{a}	2.77 ± 3.53
	200	58.4 ± 3.37^{a}	0.37 ± 6.82	107.7 ± 3.28^{a}	3.76 ± 2.38
	300	56.4 ± 3.06^{a}	3.78 ± 2.06	108.1 ± 3.54^{a}	3.47 ± 1.99
10% LRP	100	55.3 ± 1.33^{b}	5.68 ± 1.43	$100.7 \pm 4.38^{*c}$	10.02 ± 6.97
	200	$47.1 \pm 1.57^{***b}$	19.69 ± 1.61	$86.4 \pm 2.45^{***b}$	22.81 ± 2.87
	300	$42.1 \pm 1.97^{***a}$	25.39 ± 0.82	$71.7 \pm 4.10^{***a}$	32.21 ± 4.88
20% LRP	100	$47.3 \pm 1.5^{***b}$	19.32 ± 2.56	$87.8 \pm 4.13^{***c}$	21.52 ± 5.97
	200	$41.6 \pm 2.91^{***ab}$	28.97 ± 7.45	$77 \pm 2.91^{***b}$	31.25 ± 3.05
	300	$37.6 \pm 1.96^{***a}$	35.79 ± 4.18	$67.6 \pm 2.34^{***a}$	39.58 ± 1.2
Whole Wheat Flour					
0% LRP	100	57.7 ± 2.02^{a}	1.52 ± 5.64	110 ± 3.41^{a}	1.78 ± 2.49
	200	57.1 ± 1.5^{a}	2.65 ± 2.23	106.6 ± 2.43^{a}	4.76 ± 3.61
	300	56.4 ± 3.09^{a}	3.78 ± 1.88	104.3 ± 2.01^{a}	6.85 ± 2.24
10% LRP	100	53.4 ± 1.32^{b}	8.90 ± 2.37	$97.3 \pm 2.78^{***c}$	13.09 ± 2.83
	200	$44.6 \pm 1.92^{***b}$	23.86 ± 3.93	$83.8 \pm 3.01^{***b}$	25.09 ± 3.27
	300	$41 \pm 2.2^{***a}$	30.11 ± 1.42	$69.1 \pm 2.78^{***a}$	38.29 ± 3.12
20% LRP	100	$42.8 \pm 1.52^{***b}$	26.89 ± 2.5	$78.3 \pm 1.69^{***c}$	30.05 ± 0.51
	200	$39.1 \pm 2.36^{***ab}$	33.33 ± 5.64	$70.5 \pm 1.84^{***b}$	37 ± 0.55
	300	$33.8 \pm 1.99^{***a}$	42.23 ± 2.23	$59.4 \pm 2.05^{***a}$	46.92 ± 1.55
Reference drug					
Diclofenac	5	$50 \pm 1.17^{***c}$	14.77 ± 2.15	$82.1 \pm 1.61^{***c}$	26.68 ± 1.17
	10	$38 \pm 1.05^{***b}$	35.23 ± 2.56	$55.4 \pm 1.44^{***b}$	50.49 ± 1.38
	15	$25.8 \pm 0.69^{***a}$	51 ± 1.32	$37.5 \pm 1.88^{***a}$	66.47 ± 2.84
Control		58.66 ± 3.31		112 ± 3.28	

*Each value is expressed as mean±SEM of sample size (n=3). Superscripts written in different alphabets within a column indicate statistically significant difference at $P \leq 0.05$ while the same within a column indicate statistically insignificant difference at $P \geq 0.05$ among doses of a single group

The paw licking time with respect to control represented by asterisks indicating significant difference at ($P < 0.05$, **$P < 0.01$ and ***$P < 0.005$) and non-significant values are without asterisks.

Table 5: Effect of Lotus Root Powder (LRP) incorporation in unleavened flat-bread (UFB) prepared with refined wheat flour (RWF) and whole wheat flour (WWF) on hot plate-induced jumping response in mice.

TREATMENTS	DOSE (mg/kg)	TIME INTERVAL (MINUTES)		
		30	60	90
		LATENCY TIME (seconds)		
Pure LRP	400	15.3 ± 0.89^{***a} (7.26)	17.1 ± 0.83^{*a} (9.07)	17.3 ± 0.52^{***a} (8.62)
	500	17.1 ± 0.67^{***ab} (19.23)	20.3 ± 0.76^{***b} (29.78)	19.8 ± 0.62^{***ab} (24.36)
	600	19.8 ± 0.66^{***b} (38.41)	22.3 ± 0.41^{***b} (42.34)	21.9 ± 0.42^{***b} (37.88)
Refined Wheat Flour				
0% LRP	400	14.3 ± 0.46^{a} (0.43)	16 ± 0.83^{a} (2.34)	16.3 ± 1.93^{a} (1.93)
	500	14.4 ± 1.02^{a} (0.5)	16.5 ± 1.39^{a} (3.25)	16.3 ± 0.59^{a} (2.21)
	600	14.5 ± 1.07^{a} (1.13)	16.2 ± 0.87^{a} (3.4)	16.7 ± 0.72^{*ab} (4.65)
10% LRP	400	14.6 ± 0.64^{**a} (2.14)	15.8 ± 0.72^{a} (1.42)	16.2 ± 0.59^{*a} (1.38)
	500	15.3 ± 0.93^{***a} (7.19)	17.5 ± 0.57^{*a} (11.56)	16.9 ± 0.87^{**a} (6.25)
	600	16.5 ± 0.51^{***a} (15.50)	19.3 ± 0.34^{***b} (23.05)	18.2 ± 0.39^{***a} (14.26)
20% LRP	400	15.5 ± 0.48^{***a} (8.35)	17.8 ± 0.5^{**a} (13.69)	16.9 ± 0.68^{**a} (6.32)
	500	17 ± 0.58^{***ab} (19.46)	20.1 ± 0.35^{***b} (28.29)	18.8 ± 0.32^{***b} (17.82)
	600	18.5 ± 0.89^{***b} (29.63)	21 ± 0.34^{***b} (34.33)	19.8 ± 0.29^{***b} (24.65)
Whole Wheat Flour				
0% LRP	400	14.4 ± 0.75^{*a} (0.82)	16 ± 0.79^{a} (2.62)	14.5 ± 1.43^{a} (1.2)
	500	14.5 ± 0.73^{*a} (1.13)	16.2 ± 0.77^{a} (3.69)	16.3 ± 0.87^{a} (3.26)
	600	15.7 ± 0.79^{***a} (9.98)	16.3 ± 0.87^{a} (3.40)	16.5 ± 0.96^{a} (2.14)
10% LRP	400	15.4 ± 0.73^{***a} (7.34)	17.8 ± 0.71^{a} (13.55)	18.4 ± 0.72^{***a} (15.45)
	500	16.8 ± 0.61^{***ab} (17.28)	20.1 ± 0.65^{***b} (28.29)	19.4 ± 0.59^{***a} (21.65)
	600	18.5 ± 1.14^{***b} (29.17)	21.1 ± 0.97^{***b} (34.54)	20.5 ± 0.64^{***a} (28.48)
20% LRP	400	16.17 ± 0.86^{***a} (13.09)	19.7 ± 0.26^{***a} (25.67)	18.8 ± 0.32^{***a} (17.88)
	500	18.1 ± 0.5^{***b} (26.53)	21.9 ± 0.47^{***b} (39.72)	20.4 ± 0.34^{***b} (28.13)
	600	19.2 ± 0.41^{***b} (33.98)	21.9 ± 0.52^{***b} (40.14)	21.3 ± 0.43^{***b} (33.49)
Reference drug				
Paracetamol	300	16.2 ± 0.58^{***a} (13.17)	19.5 ± 0.57^{***a} (24.63)	17.6 ± 0.47^{***a} (10.29)
	400	21.5 ± 1.29^{***b} (50.06)	22.6 ± 1.23^{***b} (44.33)	20.5 ± 0.57^{***b} (28.83)
	500	23.3 ± 0.76^{***b} (62.87)	24.3 ± 0.31^{***b} (55.35)	21.9 ± 0.77^{***b} (37.39)
Control		14.3 ± 0.59	15.6 ± 0.76	15.9 ± 0.59

*Each value is expressed as mean ± SEM of sample size (n=3). Superscripts written in different alphabets within a column indicate statistically significant difference at $P \leq 0.05$ while the same alphabets within a column indicate statistically insignificant difference at $P \geq 0.05$ among doses of a single group.
Percent protection is represented within parenthesis and calculated with respect to control at different time intervals.
The mean latency time with respect to control is represented by asterisks indicating significant difference at (P<0.05, **P<0.01 and ***P<0.005) and non-significant values are without asterisks.

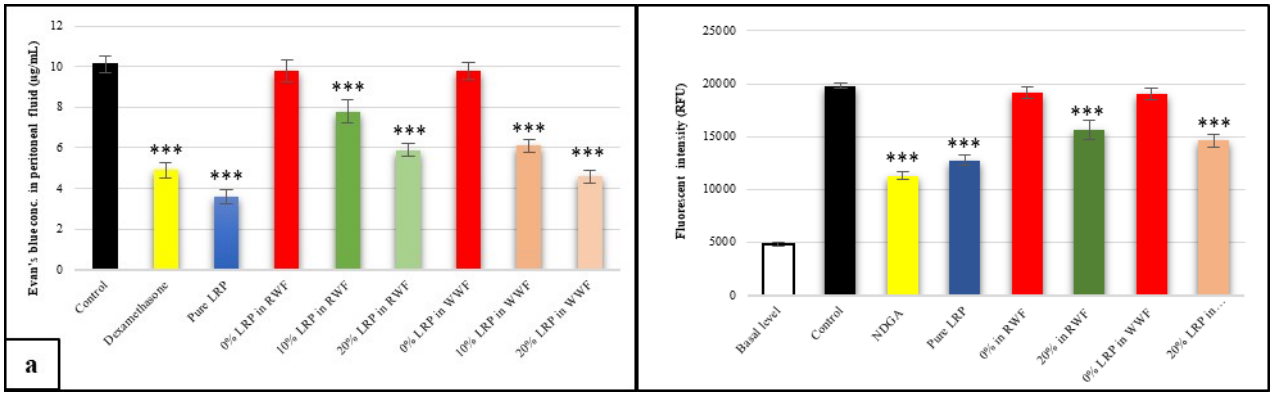

Fig. 2: Anti-inflammatory activities (a): Carrageenan induced peritonitis of UFB(s) prepared with LRP supplementation in RWF (0%, 10%, 20%) and WWF (0%, 10%, 20%) at 500mg/kg body weight, (b): Reactive oxygen species (ROS) formation in mice of UFB(s) prepared with LRP supplementation in RWF (0%, 20%) and WWF (0%, 20%) at 500mg/kg body weight. *Each bar is expressed as mean ± SEM of sample size (n=3). The % inhibition with respect to control represented by asterisks indicating significant difference at (*P<0.05, **P<0.01 and ***P< 0.005) and non-significant values are without asterisks. (One-way ANOVA, LSD test).

RWF and WWF. Decrease in ROS levels by the addition of LRP might be due to the higher % inhibition of pure LRP i.e., 35.6%.

Significant inhibition in ROS formation is reported in ethanolic extract of ginger (100 and 200µg/ml) (Akimoto *et al.,* 2015) and aqueous pomegranate peel extract at 50 ng/ml (Bachoual *et al.,* 2011).

Writhing test is considered as a quick and rapid peripheral analgesic test (Chen *et al.,* 2008) and it is also known as abdominal contortion test. Free arachidonic acid releases from phospholipids membrane as a result of acetic acid injection, thus releases prostaglandin (Alam *et al.,* 2008). It was observed that increase in the LRP ratio increased the % inhibition of abdominal writhes in mice, might be due to the suppression in pro-inflammatory cytokine TNF-α, leukotriene LTB4 and prostaglandins PGE levels in the presence of LRP (Rao *et al.,* 2007). The analgesic activity of baked products has not been found in literature; however, it is reported that lotus rhizome shows anti-inflammatory activities (Mukherjee *et al.,* 1997; Sheikh, 2014). Similar researches were reported, in which taraxeren-3-one; the active component of *D. mespiliformis* (Chang *et al.,* 2011) and extracts of *D. Lotus.* showed the prominent reduction in abdominal constriction (Rauf *et al.,* 2014).

The formalin administration immediately affects and increase afferent C-fiber; thereby evokes the pain (Heapy, 1987). LRP incorporation (20%) in RWF in late phase and 20% LRP incorporation in WWF in both early phase and late phase showed higher paw licking reduction at 300 mg/kg than that of pure LRP. This proved the synergistic effect of wheat flours and LRP, which showed higher analgesic affect. Analgesic effects of edible commodities were reported, in which extract of whole fruit of *Lagenaria breviflora* showed dose dependent reduction in formalin induced paw licking in both early (88.17-72.33 sec) and late (72.50-35.83 sec) phases (Onasanwo *et al.,* 2011). Likewise, the extract of *Microcospaniculata* fruit also showed a similar pattern (Aziz, 2015).

Hot plate test is an important analgesic test which causes different behaviors in mice as the heat activates nociceptors by impulse transmittance of the spinal cord's dorsal horn and cortical centers (Chapman *et al.,* 1985). The results evaluated the significant increase in latency time in dose dependent manner at 400-500mg/kg at 20% LRP incorporation in both RWF and WWF. The highest % protection of test agents was observed on 60th minute at 600mg/kg in both the cases. In previous studies, hot plate latency tests after oral treatment of some edible fruits were analyzed, such as limonoids from *Melia toosendan* fruit (Xie *et al.,* 2008) and *Terminalia chebula* fruit, in which the maximum latency time was observed on 30th minute at both 250 and 500mg/kg body weight (11.1±1.3 and 9.2±1.1 seconds respectively) (Ibne Jamil *et al.,* 2014), but unveiling the analgesic activities of other baked products requires further investigation.

CONCLUSION

In conclusion, the supplementation of UFB(s) with LRP showed enhanced nutritional properties, antioxidant activity and total flavonoid content as compared to the only wheat flours UFB(s). The *In Vivo* anti-inflammatory and analgesic properties proved that the incorporation of LRP helped in preventing the inflammation and pain in mice, without producing any toxic effects. Furthermore, UFB(s) prepared with WWF exhibited higher nutraceutical properties than of RWF. Hence, the current study unveils that LRP supplementation in wheat flours enhances the functional properties of UFB(s) and helps in reducing the risks of various chronic diseases.

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