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Antibacterial effect of Royal gelly, mix from Royal jelly and rape honey (1:100), rape and oak honeydew honeys against *Escherichia coli* (ATCC 25922)

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The Royal jelly (RJ), RJ and rape honey (HJ), rape (RH) and oak honeydew honey (HH) at concentrations of 10 to 45% were contaminated with 50 to 100 CFU/ml *Escherichia coli* ATCC 25922. After plating onto ChromoCult® TBX agar up to 30 min from contamination and after enrichment in Tryptic soy broth (TSB) at 37°C for 24 h, it revealed no visible colonies. These concentrations were defined as real bactericidal concentrations (RBC). Judging by results from spectrophotometric method, percent of inhibition and pH, it was observed, follow succession from drop of antibacterial activity for tested substances that RJ > HH > HJ > RH.

Key words: Royal jelly, honey, antibacterial, *Escherichia coli*.

**INTRODUCTION**

The use of honey as a traditional remedy for microbial infections dates back to ancient times (Molan, 1992). The difference in antimicrobial potency among the different types of honeys can be more than 100-fold, depending on its geographical, seasonal and botanical source as well as through harvesting, processing and storage conditions (Molan and Cooper, 2000).

The antimicrobial activity of honey is attributed largely to osmolality, pH, hydrogen peroxide production and the presence of other phytochemical components. In vivo, such activity may occur due to a synergistic relationship between any of these components rather than a single entity (Mavric et al., 2008). It was found that the honey acids and low pH exert the main antibacterial factors (Bogdanov, 1997). Manuka honeys from New Zealand, which originates from the manuka tree (*Leptospermum scoparium*), is sold as a therapeutic antibacterial agent world wide. The presence of methylglyoxal (MGO) in this type of honey has been termed as unique manuka factor (UMF®) (Willix et al., 1992; Taormina et al., 2001).

Usually in spectrophotometrical studies for detection of antibacterial effect of honey, turbidity of solutions are connected, contaminated with microorganisms with high count of bacteria and as a result, there are no counted microorganisms from them. The minimum inhibitory concentration (MIC) described the concentration before the tubes with turbid solutions and all concentrations greater than MIC are described as bactericidal concentrations.
Pathogen strains of *Escherichia coli* are often causative agents of more intestinal infections for animals and humans. According to spectrophotometric studies, the MIC<sub>100</sub> (the lowest concentration of test material which results in 100% inhibition of growth) value for *E. coli* to manuka honey was 12.5% (v/v) (Patton et al., 2006).

Some authors found that growth of *E. coli* was completely inhibited by 30 to 100% honey concentrations (Noori and Al-Waili, 2004). In other study, antibacterial activity of 13 types of honey were tested at four concentrations (10, 2.5, and 1% w/v) against *E. coli*. It was found that several honeys can inhibit *E. coli* and may have potential as therapeutic honeys (Wilkinson and Cavanagh, 2008). As the potential role for honey as a topical agent to manage surgical site or infections is increasingly acknowledged, other types of honeys need to be assessed and evaluated (Gethin and Cowman, 2008).

The hypopharyngeal glands of the honeybee (*Apis mellifera L*) produces royal jelly (RJ) that is essential to feed and raise broods and queens (Li et al., 2010). RJ may cause allergic reactions in humans, asthma and even fatal anaphylaxis; thus this product remains unaffordable in most countries (Leung et al., 1997; Lombardi et al., 1998; Takahama and Shimazu, 2006). From another perspective, it was found to have more positive effect of RJ: immunostimulating, activating vegetative and central neural systems etc. The main RJ acid, 10-hydroxy-2-decenonic acid (10-HDA) is known to have high antibiotic effect (Blum et al., 1959; Mellou and Chinou, 2005). Research suggests that the 10-HDA found in RJ may inhibit the vascularization of tumors (Izuta et al., 2007). Recently, it was found to have specific antibacterial peptide Royalisin in RJ and displayed certain antibacterial activities against Gram-positive bacteria (Shen et al., 2010). Some authors work on peptides originally isolated from the RJ and on their analogs. They found synergy with peptides belonging to the family of temporins (temporin A and temporin B) against *Staphylococcus aureus* A170 and *Listeria monocytogenes* (Romanelli et al., 2011). Few studies found out about antibacterial effect of RJ on Gram-negative microorganisms (Shirzad et al., 2007). Recently, *E. coli* have been used to determine the minimum inhibitory concentration (MIC) of a freshly reaped RJ. The MIC of RJ against *E. coli* was 2% (v/v) (Boukraa et al., 2009). To avoid acid taste and allergic reactions after consumption of royal jelly, many producers recommend mixing of this product with honey, mainly in proportion of 1:100. In available references not cited, studies about exact degrees of antibacterial activity from this mix have been conducted. There are no comparative investigations between spectrophotometrical and microbiological methods for MIC determination for RJ and mix from RJ, with different types of honey.

Thus, the aim of our study was to investigate, by microbiological method, the real bactericidal concentration (BRC) or 100% inhibition (0 CFU/ml), after 24 h incubation by 37°C, of royal jelly (RJ), mix from royal jelly and rape honey (HJ), independent used rape (RH) and honeydew (HH) honeys to referent strain of *E. coli* (ATCC 25922). Also, to compare calculated % of inhibition by parallel implemented microbiological and spectrophotometrical method.

**MATERIALS AND METHODS**

**Test substances**

Used in the experiment, RJ was obtained from “Beekeeping Centre-South”, town Stara Zagora, Bulgaria. Investigated RH was obtained in the period of May to June, 2011 from the village Kozarevec, region of Stara Zagora, Bulgaria. Mixtures from RJ and RH (HJ) in proportion 1:100 w/w (1.0 royal jelly: 100.0 rape honey) have been done experimentally. Used in the study, HH was obtained in a period of July to August, 2008 from apiary near Madzharovo, Haskovo region, Bulgaria. Prior to analyses, all honey samples were stored in dark place, with storehouse conditions (up to 18°C). The samples (bee honey and royal jelly) were from bee families not treated with sugars or antibiotics. Samples (bee honeys) were received by unshealing of combs, centrifugation and filtration. As used in the study, RJ was pipette directly from queens cells. The analysis of RJ samples was published in Balkanska et al. (2012). In brief, the following parameters were determined: proteins by Folin-Ciocalteu reagent; sugars (fructose, glucose, sucrose) by high performance liquid chromatography (HPLC); water content by refractometer; pH, potentiometrically; total acidity by titration with 0.1 N NaOH; electrical conductivity by conductimeter.

pH and free acidity were applied by the European requirements (Bogdanov et al., 1997; Codex Alimentarius Committee on sugars, 2001; European Commission, 2002). The botanical origin of the samples was established by their melissopalynological, organoleptic, physical and chemical characteristics (Table 2) (von der Ohe et al., 2004; Oddo et al., 2004). From some authors, only storage of RJ in frozen state prevents decomposition of biologically active proteins and thus RJ should be frozen as soon as it is harvested (Li et al., 2007). For our experiments, RJ was stored prior to analysis in the dark bottle in frozen conditions (-10°C). Immediately before conducting microbiological assays in order to aid pipetting during preparation of diluted honey solutions, all test substances were adjusted to 40°C in a water bath. Solutions containing 10, 20, 30, 40 and 45% (v/v) from each of test substances were prepared in sterile TSB. To prevent photodegradation of glucose oxidase, connected with antimicrobial activity in honey (Bogdanov, 1997), all honey samples and HJ were stored at room temperature in the dark and dilutions were prepared immediately prior to testing (Sherlock et al., 2010).

**Bacterial strain and preparation of bacterial suspension**

Bacterial suspension was with density 0.5 McFarland and prepared from 24 h bacterial culture of referent strain *Escherichia coli* ATCC 25922, by taking 3 to 4 colonies and dissolving in 0.85% sterile saline solution. Received bacterial suspension was with approximate concentration 1.5 × 10<sup>8</sup> CFU/ml. From suspension were prepared tenfold dilutions with sterile Triptic soy broth (TSB), (Merck) at to 10<sup>7</sup>. For detection of exact count of *E. coli* from each of...
dilutions (1 ml) was made cultivation with ChromoCult® TBX Agar (Merck), followed by incubation with 37°C for 24 h. By microbiological examination from bacterial suspension and dilutions it was found that in dilution used for contamination of test substances, E.coli were in concentration of 1.1 × 10^9 CFU/ml.

**Determination of percent inhibition (%) by spectrophotometric assay**

A spectrophotometric assay through Vis Spectrophotometer (Model: SP-870 Plus) method described by Patton et al. (2006), with some differences, was performed. Briefly, 0.6 ml of start bacterial suspension (1.1 × 10^9 CFU/ml) was added to 11.4 ml from each dilution of the concentrations stated (10 to 45% v/v) from all the test substances. Test tubes were incubated in the dark at 37°C for 24 h.

The optical density was determined just prior to incubation (TO) and again after 24 h incubation (TDO) at 620 nm. The optical density (OD) from each of solutions at TO was subtracted from the OD for each replicate at TDO. The percent inhibition of growth was determined using the formula:

Percent inhibition = 1 - (OD test tube/OD of corresponding control tube) × 100

**Determination of percent inhibition (%) and real bactericidal concentration (RBC) by microbiological assay**

Used quantity of 0.6 ml for test suspension of E. coli in each test solution (11.4 ml) maintained the mean final concentration of 55 CFU/ml from all dilutions in TSB contaminated with bacterial suspense test substances and possessed different acidity (Table 1). By parallel microbiological assay it was found that in positive control (0.6 ml from used dilution from bacterial suspense to 11.4 ml TSB), count of E. coli increased after 24 h incubation from initial 55 CFU/ml to 1.1 × 10^10 CFU/ml. To determine whether the antibacterial activity of all tested substances was bacteriostatic or bactericidal, 1 ml from each test-tube with each concentration of test substances was plated in 2 Petri dishes, poured with about 15 ml selective agar (ChromoCult® TBX Agar) (Merck), followed by incubation at 37°C for 24 h and counting of visible colonies (Table 2).

To compare with spectrophotometric method and to establish time depending influence from test substances to the growth of E. coli, this microbiological survey was done parallel with spectrophotometric assay twice, up to 30 min after inoculation (without incubation), and after 24 h incubation at 37°C from all dilutions in TSB of contamination with bacterial suspense test substances. Similar methodology was used in previous study but with Ancomonas hydrophilus as test microorganism (Strat et al., 2012). The concentration of test substances with used dilution from bacterial suspension in TSB, which causes lack of visible colonies onto selective TBX agar up to 30 min from contamination and after incubation for 24 h at 37°C, was defined as the real bactericidal concentration (RBC) (Strat et al., 2012).

With a view to calculate percent of inhibition by microbiological method we adopted 100% as the initial (55 CFU/ml up to 30 min) and final (1.1 × 10^9 CFU/ml after 24 h incubation) bacterial count in positive control without antibacterial substances. Having in mind the exact counts of colonies before and after incubation, from each of test substances was calculated the percent of inhibition. RBC and percent of inhibition by microbiological method were compared with percent of inhibition found by spectrophotometric method (Table 2). All experiments were done in the "Department of Hygiene, Technology and Control of Animal Foodstuffs, Veterinary Legislation and Management", Trakia University, Stara Zagora, Bulgaria.

**Table 1. pH and free acidity of royal jelly, honey samples and positive control in TSB before inoculation.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RJ</th>
<th>HJ</th>
<th>RH</th>
<th>HH</th>
<th>TSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.98</td>
<td>5.23</td>
<td>5.41</td>
<td>4.99</td>
<td>7.3</td>
</tr>
<tr>
<td>Free acidity</td>
<td>62</td>
<td>55</td>
<td>32</td>
<td>40</td>
<td>-</td>
</tr>
</tbody>
</table>

RJ – Royal jelly; HJ –Royal jelly mixed in rape honey 1:100 w/w; RH – rape honey; HH – oak honeydew honey; TSB – Triptic Soy Broth.

**RESULTS**

Results found by microbiological method showed that after a period from 30 min for adaptation of bacterial cells, their count by 10% concentration from all test substances remained almost constant (Table 2). Concentrations above 20% had influence on the count of E.coli and this effect was great for RJ, where count was 3 CFU/ml (94.6% of inhibition) and by high concentrations (30, 40 and 45%), cultivated forms of bacteria was not found (100% of inhibition). Despite this, we have in mind a short time for influence on microorganisms; in this case we cannot consider this effect as bactericidal. For HJ, RH and HH, cultivated forms of E.coli was found from 20 to 40%, but its count was lower than 10% concentrations. After a period for incubation of test substances by 37°C for 24 h was count bacterial growth, but with different intensity from each test substances and concentrations (Table 2).

Received results from microbiological assay for 10% concentrations from all tested substances showed high quantity of bacteria above 10^5 CFU/ml (>40% inhibition). Similar results were found by 20% RJ and HH. For RJ and HJ, 20% concentration lead to inhibition of bacterial growth and this was more intensive for RJ. Detected count of bacteria for RJ was only 7 CFU/ml (93.6% inhibition), and for HJ it was 2.0 × 10^5 CFU/ml (46.5% inhibition). For concentrations 30, 40 and 45%, RJ had lack of bacterial multiplication and 100% inhibition of bacterial cells, which demonstrated the real bactericidal effect from substance (RBC). Similar tendency was found for same concentrations of HH.

For 20%, HJ found increased bacterial count but not with normal speed and with long generation time which lead to low bacterial count in comparison with 20% concentration of tested types of honey and 10% HJ. Low bacterial increasing was found by 30% (97.9% inhibition), HJ. These data was close to percent of inhibition found by spectrophotometrical method, respectively (99.8 and 99.9%) by 30 and 40% HJ. We can note 100% of inhibition by 45% HJ, as well as microbiological and spectrophotometrical method (Table 2). In concentrations of 30, 40 and 45% RH and 30, 40% HJ was found high % of inhibition and low count cultivated forms of E.coli whose number remained almost constant in comparison with this before incubation. This meant that bacterial cells...
Table 2. Antibacterial effect of different substances on E. coli ATCC 25922.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>% (v/v)</th>
<th>Spectrophotometrical method</th>
<th>Microbiological method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 min</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td>% inhibition</td>
<td>CFU/ml</td>
<td>% inhibition</td>
</tr>
<tr>
<td>RJ</td>
<td>10</td>
<td>74.2</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>95</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>97.2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>96.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>96.3</td>
<td>0</td>
</tr>
<tr>
<td>HJ</td>
<td>10</td>
<td>83.9</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>98.7</td>
<td>3</td>
</tr>
<tr>
<td>RH</td>
<td>30</td>
<td>99.8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>99.9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>HH</td>
<td>10</td>
<td>51</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>66.4</td>
<td>5</td>
</tr>
<tr>
<td>Positive control (TSB)</td>
<td>55</td>
<td>0</td>
<td>1.1×10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

RJ – Royal jelly; HJ – Royal jelly mixed in rape honey 1:100 w/w; RH – rape honey; HH – oak honeydew honey; TSB – Triptic Soy Broth; RBC – Real Bactericidal Concentration; * - inhibitory effect calculated in percent (%) as a value difference on a basis CFU/ml in positive control.

were alive and vital but not in condition to breeding by these concentrations.

**DISCUSSION**

Our findings for RBC dependence from pH for tested types of honeys were in agreement with pH as the main antibacterial factor (Bogdanov, 1997). The high inhibition effect from 30, 40 and 45% RH and 20, 30 and 40% HJ could be connected with high concentrations of solutions, and for HJ also from additional antimicrobial substances from RJ (Shirzad et al., 2007). Our data for comparisons between percent of inhibition, found by microbiological and spectrophotometrical method, was not proven for all tested types of honey and RJ opinion from some authors for strong dependence between turbidity of contaminated honey solutions and high count of live microorganisms (Wahdan, 1998). Comparison with RBC for 30 to 45% RJ and HH with high turbidity and calculated high percent of inhibition, found by spectrophotometrical method, proved our opinion (Table 2). This lead to the conclusion that RH and HH turbidity of contaminated test solutions with high quantity of E. coli could not be connected with lack of real bactericidal activity. On the other hand 40 to 45% RH turbidity of contaminated test solutions could be connected with high quantity of fast crystallized glucose in this type of honey (Devillers et al., 2004). These findings could be connected also with data found by spectrophotometrical method for Manuka Care 18+ (UMF) honey, contaminated with E. coli NCIMB 8545. According to this study, the plots obtained for the bacterial species using the spectrophotometric method have a distinct nonlinear relationship (Patton et al., 2006). According to some authors, there is evidence of a two-stage process of inhibition, something that requires further investigation (Snow and Manley-Harris, 2004).
Recent studies for quality of RJ showed that in this product found high quantity of proteins (9 to 18%) (Sabatini et al., 2009). In connection with these amino acids, particularly L-proline, were with high concentrations in RJ (369 to 1930 μg/g) (Saito et al., 2011). It is well known that honeydew honeys contain more proline than blossom honeys (Bogdanov et al., 1997). This could be connected with findings that precipitate formation in honeys with high amount of protein can be explained by a well known interaction of hydrogen bonding between hydroxyl groups of phenolic compounds and peptide bonds of protein in forming strong insoluble polyphenol-protein complex in aqueous solution, resulting to high turbidity (Hategekimana et al., 2011). RJ shows high antibacterial activity toward *E. coli* ATCC 25922, which could be connected with its high acidity mainly from 10-hydroxy-2-decenoic acid (10-HDA), with high antibiotic characteristics (Blum et al., 1959; Melliou and Chinou, 2005).

Small differences between percent of inhibition, found by microbiological and spectrophotometric method for 30 to 45% RJ and HH, could be explained from data obtained by other studies. It was found that proteins with high affinity for binding polyphenols are those containing proline and from this the relative haze-forming activity depends on its proline content (Siebert, 1999, 2006). This findings lead to an hypothesis that small differences for percent of inhibition between RJ and HH by two used methods could be explained with high quantity of proline such that binding polyphenols lead to high turbidity and consequently resulted in the calculated low percent of inhibition by spectrophotometric method.

Considering the great potential of royal jelly and honey for treatments of different bacterial diseases, in the future, it is essential that research should continue and statistical comparisons between data obtained with microbiological and spectrophotometrical methods for detection of real antibacterial activity be made.

**Conclusion**

The antibacterial activity from RH with added RJ (1:100) increased and for 20% solution reached 46.5% inhibition (2.0 × 10⁶ CFU/ml). Small differences between percent of inhibition, found by microbiological and spectrophotometric method, for 30 to 45% RJ and HH could be explained by findings that proteins containing proline have high affinity for binding polyphenols and depending on proline content, which is the relative haze-forming activity (Siebert, 1999, 2006). On the basis of our results it was observed follow succession from drop of antibacterial activity for tested substances that: RJ > HH > HJ > RH. From this we could conclude that the basic factor which determined the antibacterial activity from test substances to *Escherichia coli* ATCC 25922 come from acidity (pH or free acidity). Future investigations to determine if the main antibacterial factor is pH or free acidity needs to be done.

**Conflict of Interests**

The author(s) have not declared any conflict of interests.

**REFERENCES**


Full Length Research Paper

Evaluation of anti-diarrheic properties of the aqueous methanolic extract of *Palisota hirsuta* leaves and its fractions using *in vivo* models

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The anti-diarrheic effects of the methanolic leaf extract of *Palisota hirsuta* (LEP) were evaluated using castor oil-induced diarrhea model and charcoal meal model to evaluate small intestinal transit (SIT). The plant material was extracted using cold maceration with 70% methanol for 48 h and concentrated *in vacuo* and was fractionated using column chromatography. LEP yielded 8.77% w/w material and seven fractions after column chromatography. LEP (100 mg/kg) significantly (P < 0.05) decreased total number of fecal output 3 h post-administration of castor oil compared with the 10 ml/kg distilled water treated group (negative control) and caused 46% inhibition of diarrhea compared with diphenoxylate (reference drug). The SIT was significantly (P < 0.05) decreased by all doses of LEP used (50, 100, 150 mg/kg), when compared with the negative control and caused 4% inhibition of intestinal fluid accumulation at 50 mg/kg compared with diphenoxylate. LEP fraction 2 (50 mg/kg) caused significant (P < 0.05) decrease in SIT compared with the negative control and caused 2% inhibition of intestinal fluid accumulation compared with diphenoxylate. The major phytochemical constituents of LEP fraction 2 were tannins. The methanolic leaf extract of *P. hirsuta* showed promising anti-diarrheic properties, possibly mediated by tannins, thus providing basis for its traditional use as an anti-diarrheic agent.

**Key words:** *Palisota hirsuta*, anti-diarrhea, small intestinal transit, fluid accumulation.

INTRODUCTION

Diarrhea is a major health problem in developing countries especially sub-Saharan Africa due to a number of different social, political and economic factors which contribute to the constant morbidity from acute and persistent diarrhea, as well as intermittent epidemics of cholera and dysentery (Hamer, 1998). The use of antibiotic in the treatment of certain types of diarrhea especially in people infected with *Escherichia coli* O157:H7 could be counterproductive due to development of haemolytic uremic syndrome (Wong et al., 2000). There are also concerns for antibiotic resistance (WHO, 2009). It is necessary to identify and evaluate the commonly available natural drugs for efficacy and safety as alternatives to the currently available allopathic anti-diarrheic drugs, which are expensive and can have serious side effects.

Various plant preparations have been used as sources of drugs for treatment of various ailments including diarrhea. The leaves of *Palisota hirsuta* have been used among the Igbo tribe of Eastern Nigeria for the treatment...
MATERIALS AND METHODS

Animals

Mature white albino mice of both sexes (23 to 34 g) were procured and housed in stainless steel cages. They were fed ad libitum with a standard laboratory animal feed (vital feed®, Grand cereal and oil meals Ltd. Nigeria), except where fasting was necessary. They were maintained in accordance with the recommendation in the guide for the care and use of laboratory animals (DHHS, NIH publication No. 85: 23, 1985). All animal experiments were conducted with the permission of the institution’s Animal Ethics Committee.

Plant

Fresh green leaves of *P. hirsuta* were collected from Obukpa in Nsukka Local Government Area of Enugu state, South Eastern Nigeria and were confirmed as *P. hirsuta* by a plant taxonomist. The leaves were dried under laboratory conditions at the temperature range of 25 to 27°C for about 10 days and pulverized to a coarse powder (mesh size 1.00 mm) using hammer mill. 382 g of the plant material was extracted by cold maceration using 70% methanol (Sigma-Aldrich Laborchemikalien GMBH, Germany) with intermittent shaking for 48 h. The extract was filtered with Whatmann No.1 filter paper and concentrated in vacuo to dryness using a rotary evaporator (Buchi Labotechnik, Switzerland) and was referred to as leaf extract of *P. hirsuta* (LEP). The percentage yield (w/w) of the extract was calculated using the formula:

\[
\text{Percentage yield (w/w) of the extract} = \left( \frac{\text{Weight of extracted material}}{\text{Weight of starting material}} \right) \times 100
\]

The LEP was separated into fractions using column chromatography (Harbourne, 1991). Briefly, Silica gel 60 G for column chromatography (Vicker, West York England) was used as the stationary phase and 10 g of LEP was adsorbed to it. The column was eluted with petroleum-ether, chloroform, ethyl acetate and methanol in ascending order of polarity as shown in Table 1. Two hundred and thirty five aliquots 10 ml column fractions were collected and spotted on pre-coated silica gel GF254 aluminum plate for thin layer chromatography (TLC) (Merck, Germany) and eluted with chloroform-methanol-ethyl acetate (1:3:1) in a small chromatographic tank to separate the various fractions, based on their relative mobility on TLC plates and color reactions with UV light (Buchi Labotechnik, Switzerland). This procedure yielded a total of 7 fractions which was used for the experiment. The fractions were concentrated to dryness using a rotary evaporator at 200 millibar at 40°C and were referred to as leaf extract of *P. hirsuta* fractions (LEPfr).

Castor oil induced diarrhea in mice

Twenty five mice were divided into five groups of five mice each. Group 1 received 10 ml/kg distilled water, group 2 received 5 mg/kg diphenoxylate and groups 3 to 5 received 50, 100 and 150 mg/kg, respectively of LEP. Thirty minutes post treatment 0.5 ml of castor oil (Bell sons and co. Southport England) was administered to all the animals. The animals were kept in individual cages and floor of which were lined with blotting paper, and the number of both normal and watery droppings were counted for each mouse every hour over a period of 4 h. Mean of the stools passed by the treated groups were compared with that of the control (Vander et al., 2007).

Small intestinal transit (SIT) and fluid accumulation (FA)

Twenty five mice were divided into five groups of five and were fasted for 16 h before the experiment. Group 1 received 10 ml/kg distilled water, group 2 received 5 mg/kg diphenoxylate, while groups 3 to 5 received 50, 100 and 150 mg/kg LEP. In addition, for the LEPfr, 27 mice of both sexes were grouped into 9 groups of 3 mice each while the controls received either 10 ml/kg distilled water or 5 mg/kg diphenoxylate, groups 3 to 9 received 50 mg/kg of LEPfr 1 to 7, respectively. One hour post treatment, a standard charcoal meal (0.5 ml of 5% activated charcoal suspension in 5% gum acacia) was administered to all the animals. The animals were sacrificed 30 min post-administration of charcoal meal under mild ether anesthesia and the intestine immediately isolated and ligated at the pyloric sphincter and at the ileo-cecal junction. The SIT (the peristaltic index) of each mouse was expressed as percentage of distance travelled by the charcoal meal relative to the total length of the small intestine from the pyloric sphincter to the ileo-cecal junction of each mouse. The FA was determined by weighing the intestine and its contents, then milking out the intestinal contents, and finally reweighing the empty intestine to determine the final weight. The difference between full and empty intestine was determined. Percentage inhibition of FA was calculated (Rao et al., 1997). The mean fecal output and the mean SIT were analyzed using one way analysis of variance and variant means were separated post-hoc using the least significant difference (LSD). Results were expressed as means ± standard error of means (SEM) and significance was accepted at the probability level (p < 0.05). The ratio of normal to watery droppings, percentage inhibition of diarrhea and percentage inhibition of intestinal fluid accumulation were expressed as percentages.

Spot phytochemical analysis of LEPfr

Phytochemical analysis of LEPfr was carried out using standard procedure (Trease and Evans, 1999). LEPfr was tested for the presence of alkaloids, flavonoids, tannins, glycosides, starch and carbohydrates.

RESULTS

The methanolic leaf extract of *P. hirsuta* yielded 8.77% w/w material which was dark green in color, pasty in
### Table 1. Solvent system for chromatographic separation of LEP.

<table>
<thead>
<tr>
<th>Pet ether %</th>
<th>Chloroform %</th>
<th>Ethyl acetate %</th>
<th>Methanol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>70</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>70</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>80</td>
<td>20</td>
<td>-</td>
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<tr>
<td>-</td>
<td>60</td>
<td>40</td>
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<td>40</td>
<td>50</td>
<td>10</td>
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<td>20</td>
<td>60</td>
<td>20</td>
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<td>-</td>
<td>-</td>
<td>70</td>
<td>30</td>
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<td>50</td>
<td>50</td>
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<td>-</td>
<td>-</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 1. Effect of LEP on castor oil-induced diarrhea in mice.
Mean ± SEM* is significant at p < 0.05 compared with 10 ml/kg distilled water.

Consistency and had a sharp pungent smell. Chromatographic separation of LEP yielded seven fractions. Spot phytochemical analysis of LEPfr2, which was the most active fraction showed the presence of tannins. The result of the castor oil-induced diarrhea in mice as shown in Figure 1 showed that there was no significant difference in the mean number of fecal output among the groups one hour post-administration of castor oil. Fecal output at this stage was only the solid type. At two hours post-administration of castor oil, there was a general
Table 2. Effect of LEP on fecal consistency in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>% solid droppings</th>
<th>% Watery droppings</th>
<th>% Inhibition of diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>41</td>
<td>59</td>
<td>56</td>
</tr>
<tr>
<td>C</td>
<td>42</td>
<td>58</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>61</td>
<td>39</td>
<td>46</td>
</tr>
<tr>
<td>E</td>
<td>19</td>
<td>81</td>
<td>0</td>
</tr>
</tbody>
</table>

A: 10 ml/kg distilled water, B: 5 mg/kg diphenoxylate, C – E (50, 100 and 150 mg/kg) LEP, respectively.

Table 3. Effect of LEP on small intestinal transit in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Small intestinal transit (SIT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml/kg b.w. dist. water</td>
<td>80.29±7.88</td>
</tr>
<tr>
<td>5 mg/kg b.w. diphenoxylate</td>
<td>55.10±9.53*</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEP</td>
<td>45.71±6.02*</td>
</tr>
<tr>
<td>100 mg/kg b.w. LEP</td>
<td>49.50±2.45*</td>
</tr>
<tr>
<td>150 mg/kg b.w. LEP</td>
<td>45.84±5.31*</td>
</tr>
</tbody>
</table>

Mean ± SEM* is significant at p<0.05 compared with 10 ml/kg distilled water.

Table 4. Effect of LEP on intestinal fluid accumulation in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>% inhibition of intestinal fluid accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (10 ml/kg, distilled water)</td>
<td>0</td>
</tr>
<tr>
<td>B (5 mg/kg, diphenoxylate)</td>
<td>0</td>
</tr>
<tr>
<td>C (50 mg/kg, MLEPH)</td>
<td>4</td>
</tr>
<tr>
<td>D (100 mg/kg, MLEPH)</td>
<td>0</td>
</tr>
<tr>
<td>E (150 mg/kg, MLEPH)</td>
<td>0</td>
</tr>
</tbody>
</table>

increase in the mean number of fecal output among the groups, but diphenoxylate (5 mg/kg b.w) significantly (p < 0.05) decreased the number of fecal outputs when compared with the LEP treated groups. There was also significant (p < 0.05) decrease in the number of fecal output in the LEP 100 (mg/kg b.w) and diphenoxylate (5 mg/kg b.w) treated groups 3 and 4 h post-administration of castor oil. Comparison of the ratio of solid to watery stool (Table 2) showed that LEP 100 mg/kg b.w produced 39% watery stools against 59% by diphenoxylate (5 mg/kg b.w) while comparison of the percentage inhibition of diarrhea among the groups showed that diphenoxylate (5 mg/kg b.w) caused 56% inhibition as against 46 and 7% by 100 and 50 mg/kg LEP, respectively.

LEP at doses 50, 100 and 150 mg/kg b.w and diphenoxylate (5 mg/kg b.w.) significantly (p < 0.05) decreased the SIT when compared with the distilled water group. The effect of LEPfr on FA (Table 6) showed that LEPfr2 and LEPfr3 caused 2 and 7% inhibition of intestinal fluid accumulation when compared with diphenoxylate.

DISCUSSION

Diarrhea is as a result of imbalance between absorptive and secretory mechanisms in the intestinal tract involving two components: motility and secretory components (Chitme et al., 2004). A good anti diarrheic agent must have significant effect on any or both of these components. Castor oil causes irritation of the intestinal mucosa via liberation of recinoleic acid and the irritation leads to release of prostaglandin which causes excessive intestinal fluid secretion and motility. Normal intestinal fluid absorption is also impaired by castor oil through inhibition of intestinal Na⁺K⁺ ATPase activity (Gaginella et
Table 5. Effect of LEPfr on small intestinal transit in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Small intestinal transit (SIT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml/kg b.w. distilled water</td>
<td>76.28±3.3</td>
</tr>
<tr>
<td>5 mg/kg b.w. diphenoxylate</td>
<td>54.44±3.8*</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEPfr1</td>
<td>76.24±9.3</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEPfr2</td>
<td>54.37±4.2*</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEPfr3</td>
<td>60.29±3.5</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEPfr4</td>
<td>68.81±10.7</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEPfr5</td>
<td>82.58±4.2</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEPfr6</td>
<td>68.50±4.3</td>
</tr>
<tr>
<td>50 mg/kg b.w. LEPfr7</td>
<td>58.42±7.5</td>
</tr>
</tbody>
</table>

Mean ± SEM* is significant at p<0.05 compared with 10 ml/kg distilled water.

Table 6. Effect of LEPfr on intestinal fluid accumulation (FA) in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Inhibition of FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (10 ml/kg, distilled water)</td>
<td>0</td>
</tr>
<tr>
<td>B (5 mg/kg, diphenoxylate)</td>
<td>0</td>
</tr>
<tr>
<td>C (50 mg/kg, LEPfr1)</td>
<td>0</td>
</tr>
<tr>
<td>D (50 mg/kg, LEPfr2)</td>
<td>2</td>
</tr>
<tr>
<td>E (50 mg/kg, LEPfr3)</td>
<td>7</td>
</tr>
<tr>
<td>F (50 mg/kg, LEPfr4)</td>
<td>0</td>
</tr>
<tr>
<td>G (50 mg/kg, LEPfr5)</td>
<td>0</td>
</tr>
<tr>
<td>H (50 mg/kg, LEPfr6)</td>
<td>0</td>
</tr>
<tr>
<td>I (50 mg/kg, LEPfr7)</td>
<td>0</td>
</tr>
</tbody>
</table>

Anti-diarrheic effect of LEP became apparent 3 h post administration of castor oil and sustained till the end of the experiment. Pretreatment of mice with LEP possibly resulted in amelioration of irritation and inflammation of intestinal mucosa induced by ricinoleic acid liberated from castor oil, thus leading to decrease in intestinal motility and secretion for an overall reduction in the rate of passage of watery stool. It is also possible that LEP activated the intestinal Na⁺K⁺-ATPase activity to enhance normal fluid absorption, thereby reducing diarrhea.

The motility and secretory activity of the gastrointestinal tract (GIT) are controlled by various neurotransmitters secreted by the enteric nervous system. Some are excitatory like acetylcholine and will lead to increased gut motility and secretion while some are inhibitory like noradrenaline and will lead to decreased gut motility and secretion (Guyton and Hall, 2001). LEP at all doses and its fraction 2 (LEPfr2, 50 mg/kg b.w) which was the most active fraction significantly slowed down charcoal meal transit in the gastrointestinal tract, showing that LEP could have inhibitory effects on the excitatory neurotransmitters in the gastrointestinal tract thus leading to relaxation of the gut muscles and slowing down motility. There was also a 4% decrease in intestinal fluid accumulation at 50 mg/kg b.w LEP and 2% by LEPfr2, pointing to mild antisecretory effect. The effects of LEP on the small intestinal transit and fluid accumulation tend to decrease with purification, signifying that other phytochemical constituents of LEP are important for effective anti-motility and antisecretory effects. It also seems that beyond 100 mg/kg, effectiveness of LEP as a gastrointestinal protectant decreases.

It has been found that anti-diarrheic properties of medicinal plants are due to tannins, flavonoids alkaloids, saponins, sterols, triterpenes and reducing sugars (Longanga-Otshudi et al., 2000; Teke et al., 2010). The major phytochemical constituents of LEP were found to be tannins, flavonoids glycosides and proteins (Anaga et al., 2009). The flavonoids, tannins or glycosides may have mediated the anti-diarrheic effects of LEP. Phytochemical analysis of LEPfr2 (the most active fraction) showed that the major constituents were tannins, therefore suggesting that the anti-diarrheic effects of P. hirsuta leaves were mediated mainly by the tannin content. Tannin containing drugs will precipitate proteins and have been used internally for the protection of inflamed surfaces of mucous membranes (Trease and Evans, 1999). The astringent actions of tannins function to precipitate microproteins on inflamed mucous membranes, thereby forming a protective layer over the mucosal lining and protect the underlying mucosa from irritants and toxins (Clinton, 2009).

Conclusion

Our results suggest that P. hirsuta possesses promising anti-diarrheic effects mediated mainly by the tannin component. The effects on the small intestinal transit and fluid accumulation suggest that the anti-diarrheic mechanism was through myo-relaxant effect than anti secretory. These effects of P. hirsuta seem to provide basis for its traditional use as an anti-diarrheic agent. We recommend further pharmacological and toxicological investigation into the anti-diarrheic properties of P. hirsuta.
leaves in other to exploit the full potential of this medicinal plant as an anti-diarrheic agent.

**ABBREVIATIONS**

LEP, Leaf extract of *Palisota hirsute*; LEPfr, leaf extract of *P. hirsuta* fraction; SIT, small *hirsuta* intestinal transit; FA, fluid accumulation.

**Conflict of Interests**

The author(s) have not declared any conflict of interests.

**REFERENCES**


Pharmacological evaluation of *Rumex vesicarius* Linn leaf extract and fractions in rabbit gastrointestinal ailment

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²Faculty of Medicine and Allied Medical Sciences, Isra University, Islamabad, Pakistan.
³Animal Husbandry in Tropics and Subtropics, University of Kassel, Germany.

The aqueous-methanolic leaf extract and fractions of the *Rumex vesicarius* L. (Rv.Cr) was evaluated for the possible presence of spasmogenic and spasmolytic constituents to rationalize its traditional uses in gastrointestinal disorders. In rabbit jejunum, *R. vesicarius* caused a dose-dependent (0.03 to 0.3 mg/ml) weak stimulatory effect on spontaneous contractions, followed by relaxation at the next higher dose (1 mg/ml). In the presence of atropine (0.03 µM), the spasmogenic effect was abolished and the relaxant effect was obtained at lower doses (0.1 to 1.0 mg/ml) shifting the dose response curves to the left. The spasmolytic effect on the spontaneous and K⁺-induced contractions in atropinized preparations was mediated at doses 0.03 to 1.0 mg/ml and 0.3 to 5 mg/ml, respectively which explained the involvement of calcium channel blocking (CCB) effect. The CCB effect was confirmed when pretreatment of the tissue with *R. vesicarius* produced a dose-dependent shift in the Ca²⁺ dose-response curves to the right in a similar manner as verapamil. Activity-directed fractionations revealed that the spasmolytic effect was concentrated in methanolic fraction, while spasmogenic activity in the aqueous fraction which was remarkably stronger than aqueous-methanolic extract. This data shows that the crude leaf extract of *R. vesicarius* L. contains spasmogenic and spasmolytic constituents mediating their effect through cholinergic and CCB actions, respectively, which explains its traditional use in the gastrointestinal disorders such as abdominal colics and cramps, constipation and diarrhoea, gastroesophageal reflux disease and irritable bowel syndrome/inflammatory bowel disease.

**Key words:** *Rumex vesicarius* Linn, spasmogenic, Spasmolytic, Cholinergic, CCB.

INTRODUCTION

Constipation and diarrhoea, colic and cramps are affecting 70% of the population worldwide (Quyang and Chin, 2004). Developing countries such as Pakistan, India and Bangladesh are commonly facing gastrointestinal disorders due to low standard sanitary conditions. Acute diarrhea is usually caused by an infectious agent, even though drugs, poisons or acute inflammatory reactions can contribute a lot (Thapar and Sanderson, 2004). Rotavirus is the major causative agent for infectious diarrhea, especially in young ones. However, other...
microbial agents viral (adenovirus, enterovirus and norovirus), bacterial (Shigella species, Escherichia coli, Salmonella species, Campylobacter and Vibrio cholerae) and parasitic (Cryptosporidium and Giardia) also disturbs the normal tone of gastrointestinal tract (Allen et al., 2004). Rumex vesicarius Linn., locally known as “Khat palak”. R. vesicarius L. has been used traditionally for ally pain of toothache, as astringent for nausea, cooling agent, anti-venom, and insect bite, and as appetizer, seeds were used for dysentery (Dymoke, 1972). In Ayurvedic system of medication, it was used as stomachic (Ahirrao et al., 2012), analgesic, anti tumor, laxative, flatulence, spleen disease, high cough, asthma, bronchitis, dyspepsia, heart troubles, alcoholism, and biliousness (Kirtikar and Basu, 1987). In Unani system of medication, it was used as analgesic, tonic, leucoderma, for scabies, and diuretic (Kirtikar and Basu, 1987). In other folk medicines, it was used to eradicate, hiccup, piles, and constipation (Hariparasad, 2011). Reptile insect, hepatoprotective, depurative, dysmenorrhoa, blood purifier, sedative, alkalinity, dyspepsia, urinary affection, chronic catarrh, renal disorders, bloody dysentery, and coronary (Madhavashetty et al., 2008), vomiting (Khan et al., 2013), antiviral, anti-diabetic, leucoderma, lymphatic glandular system disease, rectal prolapsus, aphrodisiac anti-cholesterol, impetigo, and carbubuncles (Nardkarnis, 2008; Pullaiah and Ali, 1999), antioxidant (Rao, 2003), stomach ache (Rao and Patil, 2012), diuretic (Rao et al., 2011), cancer and inflammation (Aggarwal et al., 2006), anti-fungal (Amira et al., 2011), antihelmintics (Rao et al., 2012), and antipyretic (Khan et al., 2013).

This study reports the spasmodic and antispasmodic activity of aqueous methanolic leaf extract of R. vesicarius Linn and its fractions.

MATERIALS AND METHODS

Plant

Indigenous medicinal plant R. vesicarius L. known by a local name of “Khat palak” belongs to family Polygonaceae. The plant was collected from the sandy fields of Monda Shahlamal District, Muzaffar Garh, Pakistan. The plant material was authenticated by expert taxonomist, Professor Dr. A. H. Dasti at the Department of Botany, Bahauddin Zakariya University, Multan, Pakistan (voucher F.P.ST-215). The plant material was made free from foreign adulterants and vegetative debris by hand picking and leaves were detached from the plant, washed and shade dried. Within 8 days, leaves became crispy. Special electrical herbal grinder was used to form coarse powder. Uniform dark green powder was obtained with characteristic smell.

Crude extract

The powdered plant material (1 kg) was subjected to maceration in 70% methanol in amber coloured glass bottle at room temperature for 8 days with occasional shaking (Harborne, 1973). The soaked material was passed through muslin cloth to remove the vegetative material and the fluid obtained was filtered through Whatman-1 Filter paper. The filtrate was evaporated on a rotary evaporator (Rotavapor, BUCHI labrotechnik AG, Model 9230, Switzerland) at 37°C under reduced pressure. Approximate yield was 11% and the extract obtained was stored at -4°C in air tight jars in lab refrigerator.

Preliminary phytochemical screening

Phytochemical screening was done for the presences of vital phytochemical classes by the method described by Tona et al. (1998).

Chemicals and drugs

All the chemicals, solvents, and drugs used were of analytical grade. Acetylcholine chloride and atropine sulfate were purchased from Ethical Laboratories Pvt. (Ltd) Pakistan. Dimethylosulfoxide, ethylenediamine tetracetic acid, glucose, magnesium chloride, magnesium sulfate, potassium chloride, potassium dihydrogenophospate, sodium chloride, sodium bicarbonate, and sodium dihydrogenophosphate were purchased from Sigma Chemical Company, St. Louis, MO, USA. Calcium chloride was purchased from Merck (Merck, Darmstadt, Germany).

Animals and housing condition

Ten adult albino rabbits (1.0 to 1.5 kg) of either sex, purchased from the local market with age limit between 6 and 7 months were used for the experiments. Animals were provided with fresh green fodder and tap water ad libitum and maintained in air conditioned room (23 to 25°C) at the Faculty of Pharmacy, Bahauddin Zakariya University, Multan. All rabbits were kept in fasting condition for at least 24 h before the commencement of experiments, but had free access to water. The experiments were approved by the Ethical Committee of the Bahauddin Zakariya University, Multan, with reference number EC/12/2012 dated 07 December 2012.

Plant extract solution

The plant extract (0.3 g) was dissolved in 1 ml of methanol to produce stock solution. From this stock solution further dilutions were made. Solutions were freshly prepared on the day of experiment.

Antispasmodic activity

We used the procedure described by Farre et al. (1991) to screen spasmyolytic activity. Contractions in the intestine portions were produced by high KCl (80 mM) to depolarize the intestine portions (Farre et al., 1991). The extracts were then applied in similar fashion to relax the tissues and percentage relaxation response on KCl induced contractions was recorded as shown in Figure 1. The following formula was used for calculations:

\[
\text{Inhibition/Stimulation} (\%) = \frac{\text{Average height of normal contraction (mm)} - \text{Average height of contractions after extract (mm)}}{\text{Average height of normal contraction (mm)}} \times 100
\]

Spasmodic activity

The extract was screened for possible cholinomimetic activity as per procedure mentioned. Tyrode’s solution was prepared having...
Figure 1. Tracing showing control (a) and the effect of crude methanolic extract of *Rumex vesicarius* leaf (Rv.Cr) on isolated rabbit jejunum preparations (b) and with atropine (c).

the following concentration (mM): KCl 2.68, NaCl 136.9, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂PO₄ 0.42, CaCl₂ 1.8 and glucose 5.55. The animals were then slaughtered and their abdomens were opened. Rabbit’s jejunum portion(s), of about 1.5 to 2 cm length, was isolated and mounted in the tissue bath containing 10 ml of Tyrode’s solution maintained at 37°C and supplied with carbogen gas (5% carbon dioxide and oxygen mixture). These portion(s) were kept in Tyrode’s solution previously aerated with the carbogen gas (Qayum, 2004). Earlier, the tissues were stabilized for normal activity for a period of about 25 to 40 min. For possible pharmacological screening on the tissues through series of experiments, methanolic leaf extract of *R. vesicarius* was tried at doses of 0.01 to 05 mg/ml. All the doses were applied in cumulative manner and the results were recorded (Farre et al., 1991). The spasmogenic and spasmylytic activity were recorded.

**Assay method**

**Isolated rabbit jejunum preparations**

The rabbit was starved over night and was sacrificed subsequent to a blow on the head. The abdomen was opened and jejunum was dissected out and cut to segments of about 2 cm in length following removal of adhering mesenteries. The segments were mounted between two stainless steel hooks in a 10 ml tissue bath, containing normal Tyrodes solution (pH 7.4), maintained at 37°C and aerated with carbogen (5% CO₂ + 95% O₂). A preload of 1 g was applied and the tissue was allowed to equilibrate for a period of 30 min during which the tissue was washed with fresh fluid at an interval of every 10 min prior to exposure to any test material. The spontaneous contractions were recorded isotonically through a Power Lab Data Acquisition System (AD Instruments, Sydney, Australia) (Gilani et al., 2005).

**Determination of Ca²⁺ channel blocking activity**

The Ca²⁺ channel blocking activity was determined by application of the methanol extract on K⁺-(80 mM) induced spastic contractions in isolated rabbit jejunum preparations (Fare et al., 1991). Isolated rabbit jejunum preparations exposed to K⁺ (80 mM) showed a sustained contraction. Extract was added in cumulative manner to demonstrate relaxation behaviour in jejunum preparation (Van-Rossum, 1963). Repeatedly, speculated that such relaxation was mediated through blockade of calcium channels (Bolton, 1979).
CCB effect of methanol extract was further confirmed by the previously reported method (Gilani et al., 2005). The isolated preparations were set to stabilized in normal Tyrode’s solution, which was subsequently substituted with K* normal, but Ca** free Tyrode’s solution. EDTA (0.1 mM) was added to remove Ca** from the tissues. Solution was further replaced with Ca** free and K* rich Tyrode’s solution. Incubated for 30 min, Ca** was added to tissue bath to get control Ca** dose-response curves (CDRCs) in a cumulative manner. The gradual increase in contractile activity of the tissue depicted that the strength of contractions was dependent on the availability of extracellular Ca** for K* induced influx of Ca**. After successive CDRCs, Ca** were found to be super imposable, and tissue was washed, and 60 min incubated for methanol extract. Then concentration response curves of Ca** were reconstructed and compared to the CDRCs. The concentration response curves for Ca** were developed in the presence of various concentrations of the methanol extract to assess a possible Ca** channel blocking effect (Bolton, 1979).

Statistical analysis

The results for spasmolytic and spasmogenic activities are expressed as the mean ± standard error of mean (SEM). EC_{50} values with 95% confidence interval were calculated using the computer software GraphPad Prism program version 6.0 for Windows (GraphPad, and San Diego, USA). Dose-response curves were analyzed by nonlinear regression sigmoidal response curve (variable slope).

RESULTS

Preliminary phytochemical screening detected the presence of tannins, phenols, saponins, anthraquinones and coumarins as constituents of the crude aqueous-methanolic extract of *R. vesicarius* (Rv.Cr), while it tested negative for the presence of alkaloid.

When tested on isolated rabbit jejunum, Rv.Cr showed spasmodic as well as spasmolytic effect. The spasmogen effect was observed at lower doses (0.03 to 0.3 mg/ml), while at the next higher dose (1 mg/ml) relaxation of spontaneous and high K’ (80 mM) induced contractions (0.03 to 5 mg/ml) with respective EC_{50} values of 1.886 mg/ml (1.576 to 2.256), 95% CI (n=4) and 1.73 mg/ml (0.9740 to 3.68, n=04) and 2.088 (1.576 to 2.767), 95% CI (n=4). Verapamil relaxed the spontaneous and high K’ (80 mM) induced contractions with respective EC_{50} values of 0.13 uM (0.104 to 0.114, n=4) and 0.013 uM (0.0096 to 0.0179, n=4).

DISCUSSION

Phytochemical analysis of crude leaf extract of *R. vesicarius* (Rv.Cr) showed the presence of saponins, tannins, anthraquinones, coumarins, phenols, and flavonoid, while the alkaloid was absent as methanolic soluble constituents (Table 1).

<table>
<thead>
<tr>
<th>S/N</th>
<th>Test</th>
<th>Observation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloid</td>
<td>No precipitation</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Saponins</td>
<td>1 cm froth</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Tannins</td>
<td>Light purple</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>Anthraquinones</td>
<td>Pink</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>Coumarins</td>
<td>Yellow fluorescence</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Phenols</td>
<td>Light purple</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>Flavanoid</td>
<td>Light yellow colour</td>
<td>Positive</td>
</tr>
</tbody>
</table>

As per Figure 1, there is moderate spasmodic activity in the absence of atropine. That means muscarinic receptors were not blocked and the extract produced stimulation on the receptors, whereas, in pretreated atropine tissues (Figure 1; with atropine), the spasmodic activity (25±88% of control, n=6, ps<0.05) was abolished. Its mechanism was attesting through muscarinic receptors because atropine is an antimuscarinic drug (Gilman et al., 1990). Thus, the left shift in EC_{50} values in the activity (EC_{50} without atropine=1.886 mg/ml (1.576 to 2.256), 95% CI, n=4) and EC_{50} with atropine=1.73 mg/ml (0.9740 to 3.68, n=4) confirm the cholinomimetic activity (Figure 2). The most interesting phenomenon was observed with and without atropine complete relaxation of the tissue preparation observed at similar dose (1 mg/ml); although, the earlier doses in the absence of atropine spasmodic activity was observed, while all phenomenon replaced by spasmodic activity in the presence of atropine. For further evaluation, tissues were depolarized with high potassium level (80 mM bath concentration) that produced a sustained contraction (Farre et al., 1991). Rv.Cr was then tried in cumulative manner to observe the spasmodic effect on the tissues. As it has been regarded in previous multiple studies that contractions induced by potassium are mediated through calcium channels by influx of calcium from extra cellular fluid and a substance which will inhibit the contraction produced by KCl is considered to have calcium channel blocking activity (Bolton, 1979; Janbaz et al., 2012; Giliani et al., 2005; Brown and Taylor, 1996). According to Figure 3, the extract produced a spasmodic effect on the KCl depolarized tissues, which attests further its calcium channel blocking activity depicted in

Table 1. Phytochemical analysis of *Rumex vesicarius* (leaf) crude extracts (Rv.Cr).
Figure 2. Effect of methanolic extract of Rv.Cr on atropinized and non-atropinized rabbit jejunum preparations. Values are mean±SEM, n=5.

Figure 3. Trace showing the effect of crude methanolic extract of *Rumex vesicarius* leaf (Rv.Cr) on K-80 induced contractions on isolated rabbit jejunum preparations.

Figure 4. Furthermore, methanolic fraction of Rv.Cr was found capable of complete relaxation at 1 mg/ml, most likely by calcium channel blockade (Figure 6). It was confirmed when it demonstrated the same pattern of activity as verapamil a standard calcium channel blocker (Figures 8 and 9). Furthermore, it shifted the calcium
Figure 4. Effect of different concentrations of methanolic extract of Rv.Cr on atropinized and non-atropinized rabbit jejunum preparation as well as effect on K-80 induced contractions. (Values±SEM, n=4).

Figure 5. Concentration dependent spasmogenic effect of Rumex vesicarius leaf extract and fractions. The responses are given as percent of acetylcholine (10 µM) induced maximum contraction. The values are shown as mean±SEM, n=6.
Figure 6. Trace showing the effect of crude methanolic fraction of *Rumex vesicarius* leaf (Me Rv.Cr) on isolated rabbit jejunum preparations.

Figure 7. Tracing showing the effect of crude aqueous fraction of *Rumex vesicarius* leaf (Aq Rv.Cr) on isolated rabbit jejunum preparations.

Figure 8. Concentration response curves showing the inhibitory effect of Me Rv.Cr on spontaneous and K+ (80 mM)-induced contractions in isolated rabbit jejunum preparations. Values shown are mean±SEM of 5 observations.
channel curves to the right same as verapamil (Figure 10a and b). In contrast, its aqueous fraction showed more aggregated spasmogenic activity (51%) than aqueous-methanolic extract (25%) by cholinergic stimulations (Figure 5, 7). Aqueous methanolic extract and methanol and aqueous fractions all are acidic in nature (Table 2) which provides pharmacological reason for its use in alkalinity.

Rv.Cr was found safe in mice up to dose 2000 mg/kg indicating safe drug relatively to others in use. This data indicates that spasmogenic activity mediated gut stimulation through muscarinic receptors, while
spasmolytic activity was through CCB.

ACKNOWLEDGEMENT

The authors are thankful to Dr. Khalid Hussain Janbaz (Dean) for his guidance throughout the experiment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES


Table 2. Nature and percentage spasmogenic and spasmylogic activity of methanolic extract of Rumex vesicarius L. and its fractions.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Spasmogenic activity (%)</th>
<th>Spasmylogic activity (%)</th>
<th>Nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv.Cr</td>
<td>25</td>
<td>99</td>
<td>Acidic</td>
</tr>
<tr>
<td>Ag Rv.Cr</td>
<td>51</td>
<td>8</td>
<td>Acidic</td>
</tr>
<tr>
<td>Me Rv.Cr</td>
<td>0</td>
<td>99</td>
<td>Acidic</td>
</tr>
</tbody>
</table>
Full Length Research Paper

In vivo antimalarial activity of crude aqueous leaf extract of Pyrenacantha staudtii against Plasmodium berghei (NK65) in infected mice

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Antimalarial activity of the crude aqueous leaf extract of Pyrenacantha staudtii was evaluated using chloroquine-sensitive Plasmodium berghei infection in mice with an objective to finding scientific evidence for the use of the plant as traditional antimalarial remedy in Ido/Osi LGA of Ekiti state, Nigeria. The crude aqueous extract of P. staudtii Engl. (Icacinaceae) (100, 200 and 500 mg/kg) was administered orally to mice infected with P. berghei in 4 days suppressive test. The antiplasmodial effect during the test of the plant in blood was determined and the extract at these doses induced 58.0 to 63.4% activity in comparison with untreated (negative) control group. Chloroquine produced 100% activity. The antimalarial activity showed by P. staudtii during the test justifies its use in traditional medicine for treating of malaria in the area.

Key words: Pyrenacantha staudtii, herb, malarial, anti-malarial, Ido/Osi, Ekiti, Plasmodium berghei, mice.

INTRODUCTION

Malaria continues to be a devastating disease, affecting millions of people living in the endemic areas in the developing world (Hopkins et al., 2007). Numerous attempts have been made to control the disease by using vector control measures and/or chemoprophylaxis, but they have had limited success (Trigg and Kondrachine, 1998). Immunoprophylaxis holds a promise, but effective vaccines are still not available. Presently, the most effective way of dealing with malaria is the administration of chemotherapeutic agents. Control of the main causative agents of malaria; Plasmodium falciparum and Plasmodium vivax, by use of the classical drugs of chloroquine and primaquine has been frustrated by the resistance of the malarial parasites to these drugs (Foote and Cowman, 1994; Borst and Ouellete, 1995; Garg et al., 1995; Collins and Jefferey, 1996). There is therefore a need to consistently searching for drug with novel modes of action to treat the disease.

Plants have been a great source of medicine useful in the treatment of various diseases (Bako et al., 2005). Therefore, to search for antimalarial drug from plant origin cannot be neglected, more especially, that the antimalarial drugs in use today (quinine and artemisinin) were isolated from plants (Gessler et al., 1994). Pyrenacantha staudtii is an annual herb found in the light tropical rain forest and farmland bushes. It is a woody...
climber with green fluorescent flowers (Falodun and Usifoh, 2006). The plant is widely distributed in south Nigeria and West Cameroon, and across central Africa to Uganda and Angola and it belongs to the family Icacinaceae (Burkill, 1985). The leaves are intensively bitter and the aqueous extract of the plant has been claimed by many traditional medicine practitioners to effectively treat many ailments including malaria, ulcer, gastrointestinal tract infections and threatened abortion (Anosike et al., 2008), dysmenorrhea and intestinal colic (Falodun and Usifoh, 2006).

*P. staudtii* is traditionally used for the treatment of blemorrhea, hernia, insomnia, intestinal pain and diarrhoea in Nigeria (Awe et al., 2011). The plant was among the plants mentioned to cure malaria among the people of Ido/Osi LGA of Ekiti State, Nigeria (Olorunniyi and Morenikeji, 2013). Since this plant is commonly used in traditional medicine to treat malaria, this experiment was initiated with an objective of investigating its antimalarial potential against *Plasmodium berghei* (NK65) in infected mice.

**MATERIALS AND METHODS**

**Plant collection, authentication and preparation**

The fresh leaves of *P. staudtii* were collected from Ajowa Farm at Ido-Ekiti in Ido/Osi LGA of Ekiti State, Nigeria. The plant was identified and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan where voucher specimen was deposited with number FHI No 108805. The leaves of the plants were air-dried inside a room and then grounded into a coarse powder. The coarse powder (200 g) was extracted using distilled water for 48 h at room temperature. The extract was filtered to obtain a filtrate which was concentrated to dryness over a water bath. Appropriate concentrations of the extract were made by serial dilution with distilled water for further experimentation.

**Malaria parasites inoculation**

Chloroquine sensitive *P. berghei* (NK65) was obtained from Malaria Drug Research Laboratory, Institute for Advance Medical Research and Training (IMRAT), College of Medicine, University of Ibadan, Ibadan, Nigeria. Parasites were maintained through serial passage in mice.

**In vivo antimalarial test in early infection (4-day suppressive test)**

Evaluation of suppressive potential of the extract was done using Knight and Peters 4-day suppressive test against *P. berghei berghei* infection in mice (Knight and Peters, 1980; David et al., 2004). Adult Swiss albino mice weighing 22 to 25 g were injected with 0.2 ml of aliquot *10⁶* parasitized erythrocytes, *P. berghei berghei* NK65 intraperitoneally (i.p.). Food and water were provided *ad libitum*. The mice were divided into groups of five per cage. On day 0 (that is, the day of infection), the crude aqueous leaf extract of the plant with the following concentrations (100, 200, 500 mg/kg/day) body weight was administered through oral route 3 h post-infection to every mouse in group 1 to 3, respectively. An initial toxicity test was conducted using the plant extract in which concentration at 500 mg/kg body weight was observed to be saved for the animals. Two control groups were set up which were groups 4 and 5. Mice in group 4 were treated with 10 mg/kg/day chloroquine body weight (Akuodor et al., 2010; Olorunniyi, 2013) to serve as positive control and mice in group 5 were kept untreated but only given water as placebo to serve as negative control. On day 1, 2 and 3, all the animals were treated accordingly (with the same dose and same route) as on day 0. Thin blood smears were prepared on day 11 post-infection. Blood films were fixed in absolute methanol, stained with Giesma stain for 25 mins at pH 7 and then microscopically examined (1000× magnification). Parasitaemia was determined microscopically by counting at least a total number of 1000 uninfected and infected erythrocytes from different fields. Percentage parasitemia was calculated as follows:

\[
\text{Percentage parasitemia} = \left( \frac{\text{No of infected erythrocytes}}{\text{Total No of erythrocytes}} \right) \times 100\%
\]

The percentage suppression of parasitaemia was expressed as mean chemosuppression and this was calculated for each dose level by comparing the mean parasitaemia in infected untreated (negative) control with those of treated mice. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups were calculated and expressed as percent reduction or activity using the following equation:

\[
\text{Activity} = 100 - \frac{\text{Mean parasitaemia treated}}{\text{Mean parasitaemia (-ve) control}} \times 100\%
\]

**RESULTS**

The suppressive activity of the crude aqueous leaf extract of *P. staudtii* against *P. berghei berghei* NK65 in infected mice was examined in early infection (4-day suppressive test). The crude aqueous extract at 100 mg/kg body weight of mice gave 61.6% chemosuppression when compared with the untreated (negative) control group on
Table 1. Antimalarial activity of crude aqueous leaf extract of *Pyrenacantha staudtii* and chloroquine in mice infected with *Plasmodium berghei berghei* NK65 in early infection (4-day suppressive test).

<table>
<thead>
<tr>
<th>Extract/drug</th>
<th>Dose (mg/kg/day)</th>
<th>Average % parasitaemia</th>
<th>% chemosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. staudtii</em></td>
<td>100</td>
<td>2.73 ± 0.67</td>
<td>61.6</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>2.61 ± 0.5</td>
<td>634</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2.99 ± 0.05</td>
<td>58.0</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Control (water)</td>
<td>0.2 L</td>
<td>7.12 ± 1.3</td>
<td>0</td>
</tr>
</tbody>
</table>

Values for parasite density are expressed as mean ± standard deviation (PD ± SD) for five mice per group and the ‘activity’ when compared with the untreated (negative) control.

DISCUSSION

Crude aqueous leaf extract of *P. staudtii* was observed to show intrinsic antimalarial activity considering its percentage chemosuppression in comparison with the untreated control group in 4-day suppressive test (Knight and Peters, 1980; David et al., 2004). Treatment of mice infected with *P. staudtii* showed no dose-dependent chemosuppression in comparison with the untreated control group unlike the results of Ajaiyeoba et al. (2006) in which the activity of methanol extract of *Annona senegalensis* depended on the doses of the extract. The highest chemosuppression observed in *P. staudtii* was 200 mg/kg/day treated group of mice. It can be deduced that increasing the concentration of the extract above 100 mg/kg body weight produced no additional suppressive effect against malarial infection. The antimalarial activity showed by *P. staudtii* could be attributable to the presence of alkaloids which was one of its constituents (Anosike et al., 2008). However, the active compound(s) known to give this activity need to be identified. The antimalarial activity showed by *P. staudtii* justifies its use in traditional medicine for treating malaria among the people of Ido/Osi LGA of Ekiti State, Nigeria (Olorunniyi and Morenikeji, 2013), where the plant was collected for the experiment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES


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The protective effect of *Panax ginseng* against chromium picolonate induced testicular changes

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Chromium occurs in the environment mainly in two states, tri (Cr(III)) and hexavalent (Cr(VI)). Chromium(III) (e.g. chromium picolinate) is essential for proper insulin function and is required for normal protein, fat and carbohydrate metabolism, and is acknowledged as a dietary supplement. In this study, we investigated the possible antioxidative properties of *Panax ginseng* against chromium picolonate (CrPic)-induced male reproductive toxicity at low and high doses. Ninety Sprague-Dawley rats were divided into six groups included the control group, *P. ginseng* group (200 mg/kg body weight), the groups treated with Cr-picolinate (0.8 and 1.5 mg/100 g body weight) alone or in combination with *P. ginseng* for 90 days. Testicular tissue and blood samples were taken for determination of epididymal sperm analysis, daily sperm production, testicular chromium, mitochondrial lipid peroxidation (LPO), hydrogen peroxide generation, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH) and ascorbic acid (Vitamin C), DNA fragmentation and plasma male sex hormones. The results indicated that the low and high doses of CrPic induced a significant alteration in all measured parameters. *P. ginseng* supplementation succeeded to restore these changes to great extent especially in low dose CrPic. It could be concluded that consumption of CrPic for a long time induced several hazards to testes. Supplementation with extra amounts of *P. ginseng* may be useful to restrain the CrPic induced testicular changes.

**Key words:** *Panax ginseng*, chromium picolonate, testicular changes.

**INTRODUCTION**

The decrease in human semen quality over the past several years is considered to be the result of deteriorating environmental conditions due to increased pollution (Jurewicz et al., 2009). However, the mechanism of reproductive toxicity of chromium is either poorly understood or unknown (Ong et al., 2002). Chromium picolinate (CrPic; chromium (III) tris (picolinate)) is primarily used by the general public for reduction of body weight and by athletes for improvement of body composition (Mirasol, 2000). Chronic CrPic supplementation results in distribution...
of chromium into a variety of tissues including the epididymal fat and testes (Hepburn and Vincent, 2003). A number of studies investigated the potential toxicity effects of CrPic, but the results are controversial. Golubnitschaja and Yeghiazaryan (2012) reported that, there have been isolated reports of deleterious effects of CrPic supplementation in rats, including decreased antioxidative enzyme activity, lipid peroxidation, and oxidative DNA damage.

Panax ginseng, a traditional multipurpose herb in Asia, has become the world’s most popular herbal supplements in recent years. Ginseng has a variety of beneficial biological processes that include anti-carcinogenic, anti-diabetic and anti-inflammatory effects, as well as cardiovascular- and neuro-protection (Jung et al., 2005). These properties of the ginseng are thought to provide many beneficial effects against organ damages (Huang et al., 2005). It has been used to important roles in maintaining oxidative status, by possessing either direct or indirect antioxidant functions (Chang et al., 2007).

The aim of the present study is to evaluate the possible antioxidative properties of P. ginseng against CrPic-induced male reproductive toxicity at low and high doses.

MATERIALS AND METHODS

Chemicals

Chromium-picolinate was obtained from Amoun Pharmaceutical Co. (El-Obour City, Cairo, Egypt). P. ginseng (Korean ginseng) was purchased from Pharco Pharmaceuticals, Alexandria, Egypt and was present in the form of syrup containing the dried roots of the P. ginseng; each ml contains 10 mg pyrogallol, sodium azide, glutathione reductase, GSH, 5,50-dithiobis (2-nitrobenzoic acid), and 2,4-dinitrophenylhydrazine were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. All other chemicals are of analytical grade.

Animals

The study was carried out on 90 young male albino rats (Sprague-Dawley) (8 to 12 weeks old) weighing 106 to 129 g and obtained from the Animals House Laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were maintained on the standard laboratory chow diet (El-Nasr Chemical Company, Cairo, Egypt) and water ad libitum. After an acclimatization period of 1 week, the animals were distributed into six groups (15 rats/group) and housed individually in stainless steel cages in a temperature-controlled (23±1°C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. This study was approved by the ethical committee of Tanta, Faculty of Pharmacy, Egypt.

Experimental design

Animals within different treatment groups (15 rats each) were treated for 90 days as follows: (I) untreated control group fed on the standard diet; (II) untreated control group fed on the standard diet plus oral P. ginseng (200 mg/kg body weight) (Ramesh et al., 2012); (III) treated orally with Cr-picolinate (0.8 mg/100 g body weight; low-dose group) (Mahmoud et al., 2006); (IV) treated orally with Cr-picolinate (1.5 mg/100 g body weight; high-dose group) (Mahmoud et al., 2006); (V) treated orally with Cr-picolinate at the low dose plus P. ginseng (200 mg/kg body weight) (Ramesh et al., 2012); (VI) treated orally with Cr-picolinate at the high dose plus P. ginseng.

Necropsy

The animals were fasted overnight, weighed, and sacrificed 24 h after the last dose by intraperitoneal injection of 60 mg/kg sodium pentobarbital. Testes and epididymides were removed and cleaned from adhering fat and connective tissues. The weights of these tissues were recorded in grams as well as g/100 g body weight. The cauda epididymides from each animal were used for sperm count and motility. The body weight of the animals was recorded on the day of initiation of the treatment and also on the day of sacrifice.

Epididymal sperm analysis

Epididymal sperm count and evaluation of the motility of epididymal sperm were done by the method of Belsey et al. (1980). The epididymal fluid was obtained by mincing the cauda epididymis in physiological saline (0.9% NaCl in distilled water) at 37°C. A 10 ml of diluted epididymal fluid was placed in Neubauer haemocytometer and total, motile, and nonmotile sperms were counted. The number of motile and non-motile sperms was determined microscopically within 5 min following their isolation from cauda epididymis at 37°C. Non-motile sperm numbers were determined, followed by counting of total sperms. The ratio of live and dead spermatozoa was determined using 1% trypan blue reagent (Talbot and Chacon, 1981). Briefly, one drop of diluted epididymal sperm suspension was mixed with one drop of 1% trypan blue solution on a microscope slide and covered with a cover slip. After incubation at 37°C for 15 min, the slides were observed under a microscope. Unstained spermatozoa were taken as viable and stained were counted as dead. Sperm viability was expressed as percentage of unstained sperm of the total sperm counted. The hypo-osmotic swelling (HOS) test was performed by combining 0.1 ml of sperm with 1.0 ml of a 150 mOsmol/L NaCl as a hypo-osmotic solution, following the method described by Jeyendran et al. (1992). After incubation of the mixture for 30 min at 37°C, sperm were observed for coiled tails under phase-contrast microscope (Olympus BX41; Olympus Optical Co. Ltd, Japan). The data were expressed as millions/ml for sperm count and for other sperm parameters, the data were expressed as percentage of total sperm.

Daily sperm production

Daily sperm production was determined in the testis of adult rats by the method of Blazak et al. (1993). Briefly, the testes were decapsulated and homogenized in 50 ml of ice cold 0.9% NaCl solution containing 0.01% Triton X-100 using a glass Teflon homogenizer. The homogenate was allowed to settle for 1 min and then was gently mixed and a 10 ml aliquot was collected into a glass vial and stored on ice. After thorough mixing of each sample, the number of sperm heads was counted in four chambers of an improved Neubauer-type haemocytometer. The number of sperm produced per gram of testicular tissue per day was calculated.

Determination of testicular chromium

0.2 g of testicular tissues was weighed for each group, placed in new 20 x 125-mm borosilicate glass culture tubes with polypropylene screw caps, and digested in a low-trace-metal
reagent grade HNO₃/HClO₄ mixture until complete dissolution and destruction of organic matter occurred. Samples were then diluted up to 20 ml final volume with deionized water (final HClO₄ content was 10% [v/v]) and were analyzed in triplicate for total Cr by inductively coupled plasma–mass spectrometry (ICP-MS) (Sutherland et al., 2000).

Isolation of testicular mitochondrial fraction
Mitochondrial fraction was isolated from the other testis by the differential centrifugation method as previously described (Latchoumycandane et al., 2002). Briefly, a 20% (w/v) homogenate was prepared in ice-cold 0.25 M sucrose solution. The homogenate was centrifuged at 1000 g for 10 min at 4°C to obtain the nuclear pellet. Mitochondrial pellet was obtained by centrifuging the post-nuclear supernatant at 10,000 g for 10 min at 4°C. The fraction was washed three times with ice cold 1.15% potassium chloride solution and finally suspended in 0.25 M sucrose solution (10 mg protein/ml). The mitochondrial fraction was used for the subsequent biochemical studies. Protein concentrations were determined using a BCA kit (Pierce, Rockford, USA) that employed bovine serum albumin as a standard.

Assay of oxidative status

Lipid peroxidation (LPO)
Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiorbarbituric acid to generate a coloured product that can be measured optically at 532 nm. A break down product of LPO, thiorbarbituric acid reactive substance was measured by the method of Buege and Aust (1976). Briefly, the stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25 N HCl and 2-thiobarbituric acid 0.37% (w/v) in 0.25 N HCl. One volume of the test sample (mitochondrial fraction) and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min on a boiling water bath. After cooling on ice, the precipitate was removed by centrifugation at 1000 g for 15 min and absorbance of the supernatant was measured at 532 nm against blank containing all the reagents except test sample. The value is expressed in μmol of malondialdehyde equivalent formed/min/mg protein.

Protein carbonyl contents
Protein carbonyl content was determined by the most common and reliable method (Levine et al., 1990) based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone. The levels of protein carbonyls were expressed as nmol of H₂O₂ generated/min/mg protein.

Hydrogen peroxide generation assay
Hydrogen peroxide (H₂O₂) generation was assayed according to Pick and Keisari (1981). Briefly, incubation mixture contained 1.641 ml of phosphate buffer (50 mM, pH 7.6), 54 μl of horse radish peroxidase (8.5 units/ml), 30 μl of phenol red (0.28 mM), 165 μl of dextrose (5.5 mM) and 100 μl of mitochondrial fraction was done at 35°C for 30 min. The reaction was terminated by the addition of 60 μl of 10 N NaOH and the absorbance was read at 610 nm against blank on a spectrophotometer. The quantity of H₂O₂ produced was expressed as nmol of H₂O₂ generated/min/mg protein at 35°C.

Enzymatic antioxidant assay

Superoxide dismutase (SOD)
SOD was assayed by the method of Marklund and Marklund (1974). Briefly, the assay mixture contained 2.4 ml of 50 mM Tris-HCl buffer containing 1 mM EDTA (pH 7.6), 300 μl of 0.2 mM pyrogallol and 300 μl enzyme source. The decrease in absorbance was measured immediately at 420 nm against blank at 10 s intervals for 3 min on a spectrophotometer. The activity of enzyme was expressed in nmol of pyrogallol oxidized/min/mg protein.

Catalase (CAT)
CAT was assayed as previously mentioned (Claiborne, 1985). Briefly, the assay mixture contained 2.40 ml of phosphate buffer (50 mM, pH 7.0), 10 μl of 19 mM hydrogen peroxide and 50 μl enzyme source. The decrease in absorbance was measured immediately at 240 nm against blank at 10 s intervals for 3 min on a spectrophotometer. The activity of enzyme was expressed in μmol of hydrogen peroxide consumed/min/mg protein.

Glutathione peroxidase (GPx)
GPx was assayed by the method of Paglia and Valentine (1967). Briefly, the assay mixture contained 1.59 ml of phosphate buffer (100 mM, pH 7.6), 100 μl of 10 mM EDTA, 100 μl of sodium azide, 50 μl of glutathione reductase, 100 μl of reduced glutathione, 100 μl of 200 mM NADPH, 10 μl of hydrogen peroxide and 10 μl enzyme source. The oxidation of NADPH was measured immediately at 340 nm against blank at 10 s intervals for 3 min on a spectrophotometer. The activity of enzyme was expressed in nmol of NADPH oxidized/min/mg protein.

Glutathione reductase (GR)
The activity of GR was assayed by the method of Carlberg and Mannervik (1975). Briefly, the assay mixture contained 1.75 ml of phosphate buffer (100 mM, pH 7.6), 100 μl of 200 mM NADPH, 100 μl of 10 mM EDTA, 50 μl of 20 mM oxidized glutathione and 50 μl enzyme source. The oxidation of NADPH was measured immediately at 340 nm against blank at 10 s intervals for 3 min on a spectrophotometer. The activity of enzyme was expressed in nmol of NADPH oxidized/min/mg protein.

Non-enzyme antioxidant assay

Reduced glutathione (GSH)
GSH was determined by the method of Ellman, (1959). Briefly, mitochondrial GSH content was estimated through suspending 0.1 ml mitochondrial homogenate with 1.7 ml 0.1 M potassium phosphate buffer (pH 8) followed by the addition of 0.1 ml Ellman’s reagent (5,5-dithiobis(2-nitrobenzoic acid). After 5 min, the absorbance was measured spectrophotometrically at 412 nm against a blank. The GSH values are expressed as nanomoles per milligram of mitochondrial protein.

Ascorbic acid (Vitamin C)
Vitamin C was assayed by the method of Omaye et al. (1979). Vitamin C was oxidized by copper to form dehydroascorbic acid which reacts with 2,4-dinitrophenylhydrazine to form the derivative bis-2,4-dinitrophenylhydrazone. This compound in strong sulphuric
acid undergoes a rearrangement to form a product which was measured at 520 nm. A mildly reducing medium with thiourea was used to prevent non-ascorbic chromogen interference. The level of vitamin C was expressed as μg/mg protein.

DNA fragmentation assays for apoptosis protocol

A distinctive feature of apoptosis at the biochemical level is DNA fragmentation. This method was used as a semi-quantitative method for measuring apoptosis. Apoptotic changes in tests were evaluated calorimetrically by DNA fragmentation and by agarose gel electrophoresis according to the procedure of Perandones et al. (1993). Testis samples were homogenized in 700 μl hypotonic lysis buffer and centrifuged for 15 min at 11,000 rpm. The supernatants (SN) containing small DNA fragments were separated; one-half the volume was used for gel electrophoresis and the other half, together with the pellet containing large pieces of DNA were used for quantification of fragmented DNA by the diphenyl amine (DPA) assay. The samples were treated with equal volumes of absolute isopropyl alcohol and 0.5 M NaCl to precipitate DNA. The samples were then washed with 500 μl of 70% ethanol and allowed to dry at room temperature. Extracted DNA was reconstituted in 12 μl of Tris-EDTA buffer and 3 μl loading buffer. The samples were incubated at 37°C for 20 min, then electrophoresed on 1% agarose gels containing ethidium bromide. At the end of the runs, gels were examined using UV trans-illumination. The DPA assay reaction was modified by Perandones et al. (1993) from Burton (1956). Briefly, perchloric acid (0.5 M) was added to the pellets containing native DNA (reconstituted in 400 μl of the hypotonic lysis buffer) and to the supernatants containing fragmented DNA followed by the addition of 2 volumes of DPA solution. The samples were kept at 4°C for 48 h. The colorimetric reaction was then measured spectrophotometrically at 575 nm. The percentage of DNA fragmentation was calculated in tests.

Determination of male sex hormones

Levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the plasma were measured using automated immunofluorescent assay-based commercial kits and a Brahms Kryptor immunoassay analyzer (Brahms LH Kryptor 820.050 and Brahms FSH Kryptor 818.050, respectively). Testosterone levels were measured using a chemiluminescence immunoassay-based commercial kit (Access testosterone 33560) and an access immunoassay analyzer (Beckman Coulter).

Protein estimation

Proteins were measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard protein.

Statistical analysis

Data are expressed as mean±standard deviation (SD) for fifteen animals per dose and analyzed statistically using one-way analysis of variance (ANOVA), followed by Tukey’s test. Probability values less than 0.05 were considered to be statistically significant against control. The SPSS Version 16.0 (SPSS, Inc., Chicago) package was used for all statistical analyses.

RESULTS

The body and organ weights after CrPic administration at 0.8 and 1.5 mg/100 g body weight (low and high dose, respectively) concentrations were displayed as shown in Table 1. Body weight gain showed significant decrease in low and high dose CrPic administered animals and also in those administered high dose CrPic plus P. ginseng (18, 30.9 and 13.5%, respectively) compared to control groups. It showed no significant change in body weight gain in animals exposed to low dose CrPic plus P. ginseng as compared to the control groups. Body weight gain showed significant increase in low and high dose CrPic plus P. ginseng groups as compared to their corresponding untreated groups. Testes (absolute and relative weights) and epididyms (absolute and relative weights) showed significant decrease in high dose CrPic administered animals and also in those administered high dose CrPic plus P. ginseng as compared to the control groups. Testes (absolute and relative weights) and epididyms (absolute and relative weights) showed significant decrease in high dose CrPic plus P. ginseng administered animals as compared to their corresponding untreated group, while those administered low dose CrPic plus P. ginseng showed no significant changes in these weights when compared with their corresponding untreated group (Table 1).

Animals administered low and high doses of CrPic showed a significant decrease in sperm count (16.2 and 25.6%, respectively), motility (20.4 and 32.9%, respectively) and daily sperm production (14.3 and 26.9%, respectively) when compared with the control groups. The addition of P. ginseng showed normalization in sperm count, motility and daily sperm production in low dose CrPic administered animals and significant decrease in animals administered high dose CrPic (17.3, 26.7 and 20%, respectively) when compared with the control groups. Sperm count, motility and daily sperm production showed significant decrease in high dose CrPic plus P. ginseng administered animals and no significant changes in low dose CrPic plus P. ginseng administered animals when compared with their corresponding untreated groups (Table 1).

Daily abnormal sperm production showed significant increase in animals administered high dose CrPic (20.3%) and no significant change in animals administered low dose CrPic, low dose CrPic plus P. ginseng and high dose CrPic plus P. ginseng as compared to the control groups. Daily abnormal sperm production showed no significant change in low and high dose CrPic administered animals when compared with their corresponding treated groups with P. ginseng (Table 1).

The hypo-osmotic swelling (HOS) test values showed
Table 1. Protective effect of *Panax ginseng* against Chromium-picolinate induced male reproductive system toxicity on the body weight and weights of the testis and epididymis of adult male rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Panax ginseng (200 mg/kg/day)</th>
<th>Chromium picolinate (0.8 mg/100 g b.w.)</th>
<th>Chromium picolinate (0.8 mg/100 g b.w.) plus <em>Panax ginseng</em> (200 mg/kg/day)</th>
<th>Chromium picolinate (1.5 mg/100 g b.w.) plus <em>Panax ginseng</em> (200 mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (b.w.) (g)</td>
<td>203.07±10.07</td>
<td>203.40±9.52</td>
<td>166.60±7.26 ab</td>
<td>198.40±9.07 ab</td>
<td>140.27±13.13 ab</td>
</tr>
<tr>
<td>Testes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute weights (g)</td>
<td>2.15±0.16</td>
<td>2.17±0.16</td>
<td>2.00±0.25 ab</td>
<td>2.13±0.16 ab</td>
<td>1.65±0.29 ab</td>
</tr>
<tr>
<td>Relative weights (g/100 g b.w.)</td>
<td>1.40±0.13</td>
<td>1.43±0.18</td>
<td>1.20±0.39 ab</td>
<td>1.25±0.35 ab</td>
<td>0.97±0.32 ab</td>
</tr>
<tr>
<td>Epididymis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute weights (g)</td>
<td>0.89±0.42</td>
<td>0.89±0.42</td>
<td>0.87±0.09 ab</td>
<td>0.86±0.08 ab</td>
<td>0.65±0.14 ab</td>
</tr>
<tr>
<td>Relative weights (g/100 g b.w.)</td>
<td>0.71±0.15</td>
<td>0.72±0.11</td>
<td>0.65±0.16 ab</td>
<td>0.71±0.10 ab</td>
<td>0.48±0.13 ab</td>
</tr>
<tr>
<td>Epididymal spermatozoa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm count (10^6/g epididymis)</td>
<td>217.86±5.59</td>
<td>218.03±6.69</td>
<td>182.47±10.03 ab</td>
<td>212.40±12.09 ab</td>
<td>162.07±7.88 ab</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>78.87±4.79</td>
<td>79.07±4.56</td>
<td>62.80±3.67 ab</td>
<td>78.20±4.28 ab</td>
<td>52.93±7.21 ab</td>
</tr>
<tr>
<td>DSP (10^6/g testis/day)</td>
<td>29.27±2.58</td>
<td>29.33±2.26</td>
<td>25.07±3.43 ab</td>
<td>28.73±1.91 ab</td>
<td>21.40±2.13 ab</td>
</tr>
<tr>
<td>DASP</td>
<td>1.82±0.47</td>
<td>1.83±0.37</td>
<td>1.97±0.36 ab</td>
<td>1.94±0.11 ab</td>
<td>2.19±0.39 ab</td>
</tr>
<tr>
<td>HOS</td>
<td>62.37±2.52</td>
<td>64.43±3.02</td>
<td>51.37±2.52 ab</td>
<td>63.60±2.80 ab</td>
<td>36.13±5.59 ab</td>
</tr>
</tbody>
</table>

Data shown are mean±SD from 15 rats per group. Data significantly different from untreated control group fed on the standard diet (group I) and untreated control group fed on the standard diet plus oral *Panax ginseng* (group II) control groups at p<0.05 are indicated by a and b respectively. Data of groups treated orally with Cr-picolinate (0.8 mg/kg b.w.; the low dose) (Group III) and (1.5 mg/100 g b.w.; high-dose group) (Group V) significantly different from their corresponding treated groups (Group IV and VI) with *Panax ginseng* at p<0.05 are indicated by c. DSP: Daily sperm production; DASP: daily abnormal sperm production; HOS: hypo-osmotic swelling test.

significant decrease in animals administered low and high dose CrPic (17.8, 20.4, 40.7 and 43.9%, respectively) as compared to the control groups. It showed significant decrease in high dose CrPic group treated with *P. ginseng* and no significant change in low dose CrPic group treated with *P. ginseng* when compared with the control groups. High dose CrPic group treated with *P. ginseng* showed significant decrease whereas low dose CrPic group treated with *P. ginseng* showed no significant change in the hypo-osmotic swelling (HOS) test values as compared to their corresponding untreated groups (Table 1).

Testicular chromium concentrations showed significant increase in animals administered CrPic low and high either alone or treated with *P. ginseng* (150, 134.9, 804.8, 826.8, 114.3, 119.5, 804.8 and 826.8%) as compared to the control groups. Testicular chromium concentrations showed no significant change in high and low doses CrPic plus *P. ginseng* administered animals as compared to their untreated groups (Figure 1). CrPic at low and high doses showed a significant increase in H$_2$O$_2$ production (20, 17.34, 49.77% and 47.78%, respectively), LPO (18.1, 14.17, 54.7 and 49.58%, respectively) and protein carbonyl contents (108.5, 115.8, 284.7 and 108.5%, respectively) in the mitochondrial fraction of testis in a dose-related manner as compared to the control groups. However, *P. ginseng* was shown to be effective in reducing H$_2$O$_2$ production, protein carbonyl contents and LPO. They showed no significant change in animals administered low
dose CrPic treated by *P. ginseng* and showed significant increase in animals administered high dose CrPic treated with *P. ginseng* as compared to the control groups. Hydrogen peroxide production, protein carbonyl contents and LPO in the mitochondrial fraction of testis showed significant decrease in low and high doses CrPic plus *Panax ginseng* compared to low and high dose CrPic administered animals (Figure 2).

Administration of CrPic at low and high doses a showed significant reduction in SOD (9.8, 9, 36.3% and 36.9%), CAT (27.0, 26.1, 40.3 and 39.5%), GPx (10.1, 9.4, 22.7 and 22.1%), GR (9.3, 8.9, 27.4 and 27.0%), GSH (19.1, 18.7, 60.9 and 60.7%) and vitamin C (36.5, 36.4, 52 and 52.1%), respectively in mitochondrial fraction of testis as compared to the control groups. *P. ginseng* improved the activity levels of SOD, CAT, GPx, GR, GSH and vitamin C levels. They showed no significant change in animals administered low dose CrPic plus *P. ginseng* and significant decrease in animals administered high dose CrPic plus *P. ginseng* as compared to the control groups. SOD (24.7 and 24%, respectively), CAT (20.7 and 19.7%, respectively), GPx (14.7 and 14%, respectively), GR (13.1 and 12.7%, respectively), GSH (41.2 and 40.9%, respectively) and vitamin C (36.5 and 36.4%, respectively) in mitochondrial fraction of testis showed significant decrease in high dose CrPic plus *P. ginseng* administered animals, whereas those administered low dose CrPic plus *P. ginseng* showed no significant change as compared to their untreated groups (Figure 2).

CrPic at low and high doses induced marked DNA fragmentation in testis (65.2, 76.4, 357.7 and 388.7%, respectively) as compared to the control groups. Animals treated with CrPic plus *P. ginseng* at high dose showed significant increase (287.1 and 206.6%, respectively), while at low dose showed no significant change in DNA fragmentation as compared to the control groups. DNA fragmentation in testis showed significant decrease in high dose CrPic administered animals and also in those administered high dose CrPic plus *P. ginseng* as compared to those administered low dose CrPic and low dose CrPic plus *P. ginseng* administered animals (Figure 2).

Serum testosterone and LH levels showed significant decrease (12.7, 13.9, 30, 30.9, 20.3, 15.7%, 33.6 and 29.8%, respectively), while FSH level showed significant increase in low and high doses of CrPic animals (19.9, 15.7, 33.6 and 29.8%, respectively) as compared to the control groups. *P. ginseng* treatment in high dose CrPic animals showed a significant decrease in serum testosterone and LH levels (17.5, 18.6, 18.8 and 14%, respectively), while at low dose showed no significant change as compared to the control groups. FSH level showed significant increase in high doses of CrPic animals treated by *P. ginseng* (35.8 and 39%, respectively) as compared to the control groups. Serum testosterone and LH levels showed significant decrease, while FSH level showed significant increase in low and high dose CrPic plus *P. ginseng* administered animals, whereas FSH levels showed significant decrease in these groups as compared to their corresponding untreated groups (Table 2).

**DISCUSSION**

Trivalent chromium supplements such as chromium picolinate and niacin-bound chromium, Cr(III) is consumed by the general population through its presence in many foods. In addition, there is widespread consumption of Cr(III) present in dietary supplements, such as CrPic, that are marketed primarily for weight loss and antidiabetic effects. Humans typically ingest 20 to 45 μg Cr(III) per day in the diet (IOM, 2001), while typical daily doses of supplements may contain 200 to 1000 μg Cr(III) (Komorowski et al., 2008).

It is known that Cr(III) can accumulate in the body. Ingestion of CrPic supplements was found to produce serum levels of chromium that were equivalent to serum levels measured in workers occupationally exposed to chromium, and to produce urinary chromium levels higher than those in people environmentally exposed to chromium. Chromium has also been shown to accumulate in rat liver, kidney, spleen, lung, gastrocnemius, testes and heart after ingestion of CrPic in the diet. The structure and coordination chemistry of CrPic may make it more toxic than other forms of Cr(III) (Stearns et al., 2002).

This study is focused to determine the comparative effects of low and high doses of CrPic-induced male reproductive toxicity and to evaluate the possible antioxidative properties of *ginseng* extract against these effects.

Trivalent chromium compounds cause toxicity at higher concentrations and/or depending on the ligands attached to it (Barceloux, 1999).

The study demonstrated that, CrPic exposure at low and high dose showed significant decrease body weight gain, testes (absolute and relative weights) and epididymis (absolute and relative weights) when compared with the control groups. The results are similar with the earlier observations where decrease in body weight due to gain in lean body mass and decrease in body fat noted in humans exposed to chromium picolinate (Gilbert et al., 1996). Hasten et al. (1997) reported a reduction in the body fat of rats supplemented with different doses of CrPic for 12 weeks without any reduction in the amount of food ingested, and attributed this finding to a possible thermogenic effect induced by the diet supplemented with CrPic, although no interaction nor treatment effects were seen for growth rate, lean body mass, or tissue weights. This could explain the lower final weights recorded for the animals that received high doses of supplementary CrPic.

Testicular weight was decreased significantly only after
only after high dose Cr exposure. In corroboration of this, testes (absolute and relative weights) and epididymis (absolute and relative weights) were decreased after Cr treatment at high dose as compared to the control groups (Chowdhury and Mitra, 1995).

It is well documented that during normal spermatogenesis, ROS are produced by the electron leakage outside the electron transfer chain (Hanukoglu et al., 1993). Oxidative damage induced by reactive oxygen species (ROS) causes tissue damage by a variety of mechanisms including DNA damage, lipid peroxidation (LPO), impaired membrane function, decreased membrane fluidity, altered structural integrity, inactivation of several membrane bound enzymes and depletion of thiols. (Gutteridge and Halliwell, 2000). Mahboob et al. (2002) reported an increase in lipid peroxidation levels in tissues were observed in all chromium picolinate-treated rats. SOD, GPx and GSH levels in the tissues were decreased in all the treated groups, while the hepatic CAT level decreased in the high dose group. Testicular LPO was markedly increased in experimental group treated with high dose Cr. However, low dose Cr treatment showed no significant alteration in LPO when compared with their respective controls. Enhanced LPO in response to Cr exposure leads to cellular degeneration along with impairment in steroidogenic enzyme activities in testis of adult rats. Normally produced ROS are neutralized by cellular antioxidant defense mechanism, which includes the antioxidative enzyme superoxide dismutase and catalase (Murugesan et al., 2005). Decline in the SOD and catalase activities after high dosage of Cr exposure indicate increased production of reactive species beyond the physiological limit. Increased oxidative stress also influences the normal functioning of Leydig cell, which plays a pivotal role in decreased testosterone production (Holcraft and Braun, 2004). Testosterone is responsible for the growth, structural integrity and functional activities of accessory sex organs as well as it helps in the maintenance of spermatogenesis (Steinberger and Steinberger, 1975). Decreased serum testosterone level in high dose Cr treated groups might be due to impaired activities of Δ5-3β-hydroxysteroid dehydrogenase (HSD) and 17β-hydroxysteroid dehydrogenase (HSD) enzymes (Chandra et al., 2007). Low serum testosterone and LH levels in high dose Cr exposed group also signifies the impairment in Leydig cell function. Serum FSH level was high in medium and high dose Cr exposed group. Therefore, it can be speculated that decreased sperm count was associated with Cr induced alteration in testicular function, which involves impaired steroidogenesis and increased serum FSH level as high serum FSH level was observed in patients with low sperm counts (Li et al., 2001). Significant effective spermatid degradation occurred after high dose Cr administration. The possible mechanism for the structural alteration in testis of Cr treated experimental animals may be due to the disruption of blood-testis barrier with consequent accumulation of Cr in the testis. The accumulation of Cr in sperm and epididymis as studied earlier (Murthy et al., 1991) further strengthen this possibility. A possible mechanism responsible morphological and functional alterations in testis could be due to the generation of ROS in response to Cr exposure (Acharya et al., 2006). The deterioration observed in the for testis is in corroboration with the increased testicular

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Panax ginseng (200 mg/kg/day)</th>
<th>Chromium picolinate (0.8 mg/100 g b.w.)</th>
<th>Chromium picolinate (0.8 mg/100 g b.w.) plus Panax ginseng (200 mg/kg/day)</th>
<th>Chromium picolinate (1.5 mg/100 g b.w.) plus Panax ginseng (200 mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum testosterone (ng/ml)</td>
<td>3.77±0.38</td>
<td>3.82±0.41</td>
<td>3.29±0.36abc</td>
<td>3.60±0.37c</td>
<td>2.64±0.42abc</td>
</tr>
<tr>
<td>Serum FSH (mIU/ml)</td>
<td>3.07±0.21</td>
<td>3.00±0.30</td>
<td>3.68±0.99abc</td>
<td>3.00±0.46c</td>
<td>5.10±1.01abc</td>
</tr>
<tr>
<td>Serum LH (mIU/ml)</td>
<td>1.28±0.07</td>
<td>1.21±0.05</td>
<td>1.02±0.12abc</td>
<td>1.23±0.08c</td>
<td>0.85±0.09abc</td>
</tr>
</tbody>
</table>

Data shown are mean±SD from 15 rats per group. Data significantly different from untreated control group fed on the standard diet (group I) and untreated control group fed on the standard diet plus oral Panax ginseng (group II) control groups at p<0.05 are indicated by a and b respectively. Data of groups treated orally with Cr-picolinate (0.8 mg/kg b.w.; the low dose) (Group III) and (1.5 mg/100 g b.w.; high-dose group) (Group V) significantly different from their corresponding treated groups (Group IV and VI) with Panax ginseng at p<0.05 are indicated by c. DSP: Daily sperm production; DASP: daily abnormal sperm production; HOS: hypo-osmotic swelling test.
lipid peroxidation along with decreased SOD, catalase activities and GSH enzyme. Beside membrane effects, LPO can damage DNA and protein, which ultimately led to sperm degradation and infertility (Sikka, 1996). These data explain our results about sperm morphology and function.

Cr induced hyperactivity of hypothalamo-pituitary-adrenal axis stimulates rapid release of corticotropin releasing hormone, corticotropin and glucocorticoids, respectively, from different part of this axis (Daliman et al., 1992), resulting a fall in plasma LH and testosterone levels. Therefore, increased adrenocortical activities might be another distinctive cause for Cr induced impaired gonadal function and spermatogenesis. It may be speculated from the present investigation that Cr might be toxic for male reproductive system at a much lower dose. The mechanism of reproductive impairment might be for the development of oxidative stress, disruption of hypothalamic-pituitary-testicular axis associated with hyperactivation of the stress signaling pathway through hypothalamic-pituitary-adrenocortical axis resulting excess corticosterone secretion. In this paper, we have demonstrated the dose-dependent effects of chromium(III) picolinate on enhanced production of ROS including H$_2$O$_2$ production and LPO, and DNA fragmentation and significant reduction in SOD, CAT, GPx and GR activity in mitochondrial fraction of testis.

In accordance with our findings, Speetjens et al. (1999) postulated that chromium picolinate is capable of generating hydroxyl radicals, which in turn can cleave supercoiled DNA. These results are consistent with other studies, which demonstrate the ability of certain chromium complexes to nick DNA in the presence of peroxide and a reductant or in cells. Chromium picolinate has also been shown to generate hydroxyl radicals from hydrogen peroxide in a pathway independent of added reductant. Due to chromium’s ligand composition and the resulting redox potential, the complex can be reduced readily by abundant biological reductants and generate hydroxyl radicals via Haber-Weiss and Fenton reactions. The enhanced production of hydroxyl radicals results in appreciable DNA damage (Speetjens et al., 1999). Cr(III) compounds are genotoxic in certain test systems, and interaction of Cr(III) with DNA has been shown to result in the formation of DNA adducts, DNA-protein crosslinks, and DNA interstrand crosslinks (Andersson et al., 2007; Reynolds et al., 2007; Mozaffari et al., 2012).

ROS induce a significant reduction in semen quality by decreasing sperm count and motility. They can also increase sperm defects and impairment of antioxidant synthesis (Hatamoto et al., 2006). This study observed a decrease in the six parameters of sperm motility, VSL, VCL, VAP and ALH due to CrPic treatment. It can induce the formation of abnormal sperm cells; the increase in sperm abnormalities indicates that CrPic induced DNA damage in germ cells leading to altered sperm morphology. Sikka (2004) has reported that peroxidation of critical thiol groups in protein can alter the structure and function of spermatozoa. The decrease in sperm count is an important factor leading to male infertility (Meistrich and Brown, 1983).

Endogenous and exogenous antioxidants may protect cells and tissues from destructive effects of ROS and other free radicals. Previous studies reported that sperm disorders can be improved by exogenous antioxidants/ROS scavengers (Hosseini et al., 2012). Generally, it is known that the effects of *P. ginseng* are due to their numerous ginsenosides. Substances identified *P. ginseng* extract include a number of ginsenosides, polysaccharides, polyphenols, some minerals, etc (Geva et al., 1996). *Ginseng* or its extracts have been reported to exhibit free radical scavenging activities and can prevent lipid peroxidation (Hosseini et al., 2012). Polyphenols have antioxidant activity similar to vitamin C and E, which can enhance fertility by decreasing the level of free-radical damage to sperm cells (Geva et al., 1996).

Generation of ROS decomposes sperm plasma membrane and is therefore responsible for loss of sperm motility, which is presumably caused by a rapid loss of intracellular ATP leading to damage in sperm flagellum. Activity of Na+-K+-ATPase is highly sensitive to ROS, thus depletion of Na+-K+-ATPase can be a good reason for the reduction of sperm motility. The existence of morphologic abnormalities and decreased sperm viability has been associated with ROS production (Kim and Parthasarathy, 1998; Fischer et al., 2003). Also, defects in the flagella, changes in motility and morphology of spermatozoa, are likely associated with infertility. Sperm cells are more susceptible to peroxidative damage, because of high concentration of polyunsaturated fatty acids and low antioxidant capacity (Vernet et al., 2004). Membrane-associated polyunsaturated fatty acids such as sperm are readily attached by ROS. Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentation, which can result in cell lysis (Fischer et al., 2003; Katoh et al., 2002). Reduction in sperm counts, the existence of morphologic abnormalities could be due to the generation of ROS by Cr(III).

Notably, *P. ginseng* was known to have protective and therapeutic effects against the testicular atrophy and other damages induced the most potent environmental pollutants toxic to reproductive organs (Kim et al., 1999). Salvati et al. (1996) also showed that men treated with *ginseng* have experienced an increase in spermatozoan number, improvement of motility, sperm viability, plasma testosterone, dihydrotestosterone (DHT), FSH, and LH concentrations. In rats fed with *ginseng* for 60 days, a significant increase of blood testosterone levels was found (Fahim et a., 1982). These spermatogenic effects of *P. ginseng* might be due to the combined effect of its many constituents, ginsenosides, polyphenol and minerals (Park et al., 2006). The results of this current study also suggest that the testicular changes induced by CrPic were significantly recovered by *P. ginseng*. 
Conclusion

It could be concluded that consumption of CrPic for a long time induced several hazards to testes. Supplementation with extra amounts of *P. ginseng* may be useful to restrain the CrPic induced testicular changes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES


