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Dissolution rate enhancement of bicalutamide by adsorption process

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The aim of the present research was to enhance the dissolution rate of poorly water soluble drug, bicalutamide by adsorption process. Bicalutamide is an antiandrogen agent used in the treatment of prostate cancer. To improve the dissolution rate of the drug, hydrophilic carrier like povidone K30 and adsorbent like magnesium aluminum silicate were used as dissolution rate enhancers. Granules of bicalutamide were prepared by wet granulation technique by using magnesium aluminum silicate and povidone K 30 either alone or in combination at different concentrations. The granules were evaluated for packing and compression properties. The granules were compressed into tablets, and different tableting parameters were investigated. The dissolution profile of the tablets was also evaluated and compared with the marketed product. From the dissolution profile, it was observed that the carrier ratio of 3:1 of magnesium aluminum silicate to povidone K 30 exhibited higher dissolution rate than the other formulations.

Key words: Bicalutamide, magnesium aluminum silicate, adsorption, povidone K 30.

INTRODUCTION

Presently, product development scientists working in the areas of drug discovery, preformulation and formulation studies are using various solubilization techniques for solving solubility problems related to the drug in their daily work. The solubility and dissolution rate of poorly water soluble drug can be altered in many ways, such as modification of drug crystal forms, addition of co-solvents, micronisation, solubilisation by surfactants, addition of adsorbents, solid dispersion, complexation with cyclodextrins (CD), etc. (Vippagunta et al., 2002; Chou and Riegelman, 1971; Leuner and Dressman, 2005; Kinoshita et al., 2002; Toshiro, 2006). Some of these techniques make use of organic solvents which are expensive and hazardous to our environment. Among all the possibilities, selective adsorption on insoluble carriers is one technique that can be applied to increase the dissolution rate dramatically. In the adsorption process, a highly active adsorbent such as inorganic clays like bentonite, magnesium aluminum silicate etc. enhance the dissolution rate of poorly water-soluble drugs by maintaining the concentration gradient at its maximum. The two reasons suggested for the rapid release of drugs from the surface of clays are the weak

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physical bonding between the adsorbate and the adsorbent, hydration and swelling of the clay in the aqueous media (Monkhouse and Lach, 1972).

Poor water-solubility of drugs is associated with slow drug absorption, leading eventually to inadequate and variable bioavailability. Bicalutamide comes under this class of poor water soluble drug for which its solubility is to be enhanced. The chemical name of the bicalutamide is \((\pm)-N-[4$\text{-}$cyano-3$\text{-}$((trifluoromethyl)$\text{-}$phenyl)$\text{-}3$\text{-}$[(4$\text{-}$fluorophenyl)$\text{-}$sulfonyl]$\text{-}2$\text{-}$hydroxy$\text{-}2$\text{-}$methyl$\text{-}$propanamide\) (Figure 1). It is a non-steroidal antiandrogen used for the treatment of prostate cancer (Michael et al., 2009). It competitively inhibits the action of androgens by binding to cytosol androgen receptors in the target tissue (Fuzheng, 2006). Bicalutamide blocks the growth stimulating effect of androgens on prostate cancer. The aqueous solubility of bicalutamide is low (5 \text{µg/ml}) when determined by in vitro method at pH 7 and 37°C (Srikanth et al., 2010a). Since the \(pK_a\) of bicalutamide is 12, hence the solubility of the drug is low at physiological pH. Thus, it is important to enhance the solubility and dissolution rate of bicalutamide to improve its oral bioavailability (Srikanth et al., 2010b).

The process of adsorption has numerous applications of diverse nature in the pharmaceutical field in formulation of various dosage forms. Surface method is one of the methods used to reduce the drug particle size by increasing the surface area available to the dissolution medium. Active adsorbents can enhance the dissolution rate of the poorly water soluble drugs by maintaining the concentration gradient at its maximum. The main reasons for the rapid release of drugs from the surface of the adsorbents are, the weak physical bonding between the adsorbate and the adsorbent, and hydration and swelling of the adsorbent in the aqueous medium (Manish et al., 2001; Harish and Madan, 2007).

In the present investigation, bicalutamide formulations were prepared by wet granulation technique using different combinational ratios of magnesium aluminum silicate and povidone K 30. Out of these concentrations, best ratio was selected based upon their efficiency to enhance the dissolution rate of the drug.

**MATERIALS AND METHODS**

Bicalutamide (BC) was a generous gift sample from Dr. Reddy’s Laboratories Ltd (Hyderabad, India). Povidone K 30 and sodium lauryl sulphate were obtained as gift samples from Orchid Health Care (Chennai, India). Magnesium aluminum silicate was obtained as gift samples from Unichem Laboratories Ltd (Goa, India). Bicalutamide commercially available tablet (Tabi, 50 mg, Manufactured by Dr. Reddy’s Laboratories Ltd, Mumbai, India) was purchased from local market. All other reagents and chemicals used were of analytical grade.

**Preparation of granules and tableting**

Required quantities of bicalutamide, magnesium aluminum silicate, povidone and lactose were accurately weighed and were sifted through sieve no. 40 (425 microns) (Table 1). The mixture was granulated with minimum quantity of water. The wet mass was dried in a rapid drier until the loss on drying (LOD) reached less than 2% w/w. Dried granules were passed through sieve no. 20 (850 microns) and were mixed with presifted crospovidone through sieve no. 40. The above granules were lubricated with presifted magnesium stearate through sieve no. 60 (250 microns) and mixed for 3 min. Lubricated granules were evaluated for flow characteristics and were compressed into tablets by using 8-station Rimek compression machine. Compressed tablets were allowed to equilibrate in a desiccator for 24 h before evaluation.

**Evaluation of granules**

**Packing property of the granules**

The packing properties were determined by measuring the difference between bulk density (BD) and the tapped density (TD) using standard procedure. In the procedure, a 20 g quantity of granule sample was placed into 250 ml clean, dry measuring cylinder and the volume \(V_0\) occupied by the sample without tapping was determined. An automated tap density tester (model C-TDA2, Campbell Electronics, Mumbai, India) was used for tapping the granules according to US Pharmacopeia (USP, 2006). After 100 taps, the occupied volume, \(V_{100}\) was also noted. The bulk and tap densities were calculated from these volumes (\(V_0\) and \(V_{100}\)) using the formula:

\[
\text{Density} = \frac{\text{Weight}}{\text{Volume occupied by sample}}
\]

The Hausner ratio was determined by dividing TD by BD, and Carr’s compressibility index (CI) (Garr, 1965) was determined using Equation 1:

\[
CI = \left\{ \left( \frac{\text{TD} - \text{BD}}{\text{TD}} \right) \times 100\% \right\}
\]

**Evaluation of tablets**

The formulated tablets were evaluated for all the physical parameters. The parameters evaluated include hardness, friability,
Table 1. Formulation of bicalutamide tablets.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicalutamide</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Magnesium aluminum silicate</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>150</td>
</tr>
<tr>
<td>Povidone K 30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>Avicel PH 200</td>
<td>41</td>
<td>38</td>
<td>35</td>
<td>41</td>
<td>38</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>Crospovidone</td>
<td>12.5</td>
<td>7.5</td>
<td>10</td>
<td>7.5</td>
<td>10</td>
<td>12.5</td>
<td>15</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>1.5</td>
<td>2</td>
<td>2.5</td>
<td>1.5</td>
<td>2</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>Total Weight (mg)</td>
<td>150</td>
<td>200</td>
<td>250</td>
<td>150</td>
<td>200</td>
<td>250</td>
<td>300</td>
</tr>
</tbody>
</table>

Determination of tablet tensile strength (T)

The hardness of ten tablets were determined individually with the Monsanto hardness tester (Brook and Marshall, 1968). The mean values of the fracture loads were recorded.

Disintegration test

The method described in the British Pharmacopoeia (2002) was followed using water maintained at 37°C as the disintegration fluid. Six tablets were used in each determination, which was carried out in triplicate and the mean results reported.

Friability test

The friability test is to evaluate the ability of the tablet to withstand abrasion in packaging, handling and shipping. Ten (10) tablets were weighed initially (w1), placed in friabilitator (Roche) and were allowed to rotate at the speed of 25 rpm for 4 min. After four minutes of this treatment or 100 revolutions, the tablets are weighed and the weight (w2) compared with the initial weight. The loss due to abrasion is a measure of the tablet friability. The friability (%) was calculated by using Equation 2:

Friability (%) = \( \frac{(w_1 - w_2) \times 100}{w_1} \) \( (2) \)

Drug content

From each batch, 10 tablets were randomly collected and powdered in a glass mortar. Accurately weighed drug equivalent of 50 mg of the powder was transferred into a 100 ml volumetric flask and dissolved in 20 ml of acetonitrile. The drug-acetonitrile solution was extracted with 1% sodium lauryl sulphate (SLS) solution with vigorous shaking on a mechanical shaker for 1 h and filtered into another 100 ml volumetric flask through 0.45 mm millipore nylon filter disc and the filtrate was made up to the mark with 1% SLS solution to obtain 500 µg/ml. 0.5 ml of the above solution was further transferred into 50 ml volumetric flask and was diluted up to the mark with 1% SLS solution to get 5 µg/ml. Absorbance of the obtained solution was measured at 272 nm against blank (1% SLS solution) using a Ultraviolet and visible (UV/Vis) spectrophotometer (Elico SL 210) and the drug content was measured by using pre-calibrated curve equation \( Y = 0.0527X - 0.0438 \). All the above physico-chemical properties were conducted on the existed marketed product ‘Tabi’ for comparison.

In vitro studies

In vitro studies were performed separately in 900 ml of 1% SLS maintained at 37 ± 0.5°C using USP XXII type II dissolution rate test apparatus at a stirring speed of 50 rpm which is official in Food and Drug Administration (FDA) dissolution methods. 5 ml aliquots were withdrawn at different time intervals up to one hour and replaced with same volume of drug free fresh dissolution medium so as to maintain sink conditions. The samples were filtered and estimated for the amount of bicalutamide dissolved by measuring the absorbance at 272 nm in ultra violet (UV) spectrophotometer (Elico SL 210). The dissolution experiments were done in triplicate. Dissolution studies were carried out on existed marketed product ‘Tabi’ in the similar way for comparison of drug release behavior.

RESULTS AND DISCUSSION

Packing and flow properties of the granules

The results (Table 2) showed the packing properties of the granules obtained by wet granulation technique. The CI values were between 18 to 24% while the Hausner ratio was between 1.2 to 1.4, indicating that the granules exhibited good flow properties (Rakhi et al., 2008).

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From each batch, 10 tablets were randomly collected and powdered in a glass mortar. Accurately weighed drug equivalent of 50 mg of the powder was transferred into a 100 ml volumetric flask and dissolved in 20 ml of acetonitrile. The drug-acetonitrile solution was extracted with 1% sodium lauryl sulphate (SLS) solution with vigorous shaking on a mechanical shaker for 1 h and filtered into another 100 ml volumetric flask through 0.45 mm millipore nylon filter disc and the filtrate was made up to the mark with 1% SLS solution to obtain 500 µg/ml. 0.5 ml of the above solution was further transferred into 50 ml volumetric flask and was diluted up to the mark with 1% SLS solution to get 5 µg/ml. Absorbance of the obtained solution was measured at 272 nm against blank (1% SLS solution) using a Ultraviolet and visible (UV/Vis) spectrophotometer (Elico SL 210) and the drug content was measured by using pre-calibrated curve equation \( Y = 0.0527X - 0.0438 \). All the above physico-chemical properties were conducted on the existed marketed product ‘Tabi’ for comparison.

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RESULTS AND DISCUSSION

Packing and flow properties of the granules

The results (Table 2) showed the packing properties of the granules obtained by wet granulation technique. The results showed that the granules were compressible by tapping. The CI values were between 18 to 24% while the Hausner ratio was between 1.2 to 1.4, indicating that the granules exhibited good flow properties (Rakhi et al., 2008).

Evaluation of the physical parameters of the tablets

The tableting parameters of the formulated tablets are presented in Table 3. All formulated tablets had hardness and friability values in-between 72 to 75 N and 0.2 to 0.5%, respectively. The formulated tablets also disintegrated within 5 min as against the stipulated official time of 15 min in the British Pharmacopoeia (2002). The drug contents for all formulations were > 97%. All the experimental formulations exhibited similar behavior compared
Table 2. Evaluation of the granules.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Bulk density (g/ml)</th>
<th>Tapped density (g/ml)</th>
<th>CI</th>
<th>Hausner ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.612</td>
<td>0.695</td>
<td>12</td>
<td>1.14</td>
</tr>
<tr>
<td>F2</td>
<td>0.643</td>
<td>0.735</td>
<td>12.5</td>
<td>1.14</td>
</tr>
<tr>
<td>F3</td>
<td>0.664</td>
<td>0.772</td>
<td>14</td>
<td>1.16</td>
</tr>
<tr>
<td>F4</td>
<td>0.648</td>
<td>0.761</td>
<td>14.8</td>
<td>1.17</td>
</tr>
<tr>
<td>F5</td>
<td>0.656</td>
<td>0.756</td>
<td>13.2</td>
<td>1.15</td>
</tr>
<tr>
<td>F6</td>
<td>0.697</td>
<td>0.792</td>
<td>12</td>
<td>1.14</td>
</tr>
<tr>
<td>F7</td>
<td>0.71</td>
<td>0.816</td>
<td>13</td>
<td>1.15</td>
</tr>
</tbody>
</table>

CI = Compressibility index

Table 3. Tableting parameters.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Thickness (mm)</th>
<th>Hardness (N)</th>
<th>% Friability</th>
<th>% Drug content</th>
<th>Disintegration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>3.7±0.45</td>
<td>75±0.14</td>
<td>0.24</td>
<td>99.2±0.5</td>
<td>3.2±0.21</td>
</tr>
<tr>
<td>F2</td>
<td>3.9±0.32</td>
<td>74±0.22</td>
<td>0.33</td>
<td>98.4±0.2</td>
<td>2.4±0.07</td>
</tr>
<tr>
<td>F3</td>
<td>4.1±0.67</td>
<td>72±0.09</td>
<td>0.45</td>
<td>99.1±0.7</td>
<td>3.5±0.03</td>
</tr>
<tr>
<td>F4</td>
<td>3.6±0.27</td>
<td>77±0.34</td>
<td>0.19</td>
<td>98.2±0.6</td>
<td>4.5±0.43</td>
</tr>
<tr>
<td>F5</td>
<td>3.8±0.54</td>
<td>75±0.59</td>
<td>0.28</td>
<td>101.4±0.8</td>
<td>4.7±0.22</td>
</tr>
<tr>
<td>F6</td>
<td>4.0±0.63</td>
<td>73±0.75</td>
<td>0.36</td>
<td>100.3±0.2</td>
<td>4.2±0.39</td>
</tr>
<tr>
<td>F7</td>
<td>4.4±0.16</td>
<td>74±0.44</td>
<td>0.31</td>
<td>100.2±0.1</td>
<td>3.9±0.11</td>
</tr>
<tr>
<td>Tabi</td>
<td>5.2±0.29</td>
<td>69±0.58</td>
<td>0.49</td>
<td>100.04±0.4</td>
<td>4.9±0.44</td>
</tr>
</tbody>
</table>

with marketed formulation.

Dissolution profiles

The dissolution profiles of bicalutamide from different formulations are shown in Figures 2 to 4. The results showed that the dissolution rate of the drug was greatly influenced by the amount of adsorbate concentration present. Dissolution rate of bicalutamide tablets increased with the presence of magnesium aluminum silicate concentration as shown in Figure 2. It was observed that as the ratio of magnesium aluminum silicate in the formulation increased, the dissolution rate increased correspondingly. From the dissolution data, it was also observed that pure drug released its active content less than 60% at the end of one hour, whereas the physical mixture with higher concentration (1:3) of drug: carrier released about 70% of the drug at the end of one hour. The bicalutamide-magnesium aluminum silicate adsorbates at 1:1, 1:2 and 1:3 (F1, F2 and F3) ratios exhibit 1.5, 1.8 and 2.2 fold increase in sequence after 10 min, 1.4, 1.7 and 1.8 fold increase in sequence after 30 min and 1.5, 1.7 and 1.9 fold increase in sequence after 60 min in the dissolution rate of the formulations compared with pure drug. Thus, presence of the adsorbate greatly influenced the dissolution rate.

The results of the dissolution data with povidone K 30 (F4, F5 and F6) as the drug to carrier in ratios of 1:1, 1:2 and 1:3 are shown in Figure 3. It was observed that dissolution rate was slightly enhanced by the increased concentration of povidone K 30. According to the results, pure drug released its active content less than 60% at the end of one hour, whereas physical mixture with higher concentration (1:3) of carrier released about 65% its drug content at the end of one hour. However, in case of tablet formulations the dissolution rate was enhanced by increased concentrations of carrier. As shown from the results, the drug release was 76% for F4, 82% for F5 and 85% for F6 at the end of one hour. When compared with adsorbate dissolution results, povidone made formulation had no much influence on the dissolution rate. So further trials were focused based on the formulation F3.

In the present formulation studies, the main target was to improve the dissolution rate to a maximum extent within a short period of time. Even though the drug release was fast and complete in formulation F3, there is
a need to further improve the drug release at initial time points (10 to 20 min) to bring about immediate relief of symptoms. So povidone as third component was incorporated into the existing formulation F3 and treated as F7.

The dissolution data of formulation F7 is presented in Figure 4. It was observed that the dissolution rate was greatly increased by 3 fold by the use of povidone in the formulation F7 causing a burst release at this point. The improvement of the dissolution rate on addition of povidone to the magnesium aluminum silicate may be attributed to its wetting and solubilising effect (Saleh et al., 1998). Dissolution rate as compared to pure drug at 10 min interval has been enhanced a lot and reached 98% within 20 min. Hence, this was considered as the optimized formulation. Marketed formulation 'Tabi' released 65% of the drug by the end of 10 min. From the dissolution profile it was observed that the optimized formulation (F7) enhanced the dissolution rate of bicalutamide greatly compared with marketed formulation Tabi.

**Conclusions**

Adsorbents play a significant role in the improvement of dissolution characteristics of poorly water soluble drugs. In the present study, the dissolution of bicalutamide has been enhanced by addition of adsorbents like magnesium aluminum silicate, hydrophilic carriers like povidone K 30 and combination of both. From the dissolution data it was concluded that dissolution rate was greatly enhanced in
the formulation F7 at the ratio 1:3:1 of drug: magnesium aluminum silicate: povidone K30, respectively compared to marketed formulation. Besides being economical, the major advantage of adsorbent based formulations is it avoids the usage of organic solvents which are major constituents in the preparation of conventional formulations. Therefore, the formulation F7 consisting of bicalutamide-magnesium aluminum silicate-povidone K30 (1:3:1) ratio was considered as the optimum ratio for the formulation and best candidate for further scale up studies which increases the solubility and dissolution rate of the bicalutamide, suggesting a possible enhancement of its oral bioavailability.

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REFERENCES

Full Length Research Paper

Biochemical and hematological alterations associated with intravenous administration of double doses of non-ionic contrast media in anesthetized dogs

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To monitor the effects of a double dose of non-ionic contrast agent administered intravenously (iv) to anesthetized dogs, on the major functions and the biochemical and hematological parameters, two non-ionic contrast agents were tested (Optiray 350 and Ultravist 370) on two lots of 15 adult male and female dogs. The administered dose was 7 ml/kg and the recommended dose was 3.5 ml/kg. Several biochemical and hematological parameters were determined at 15, 60 min and 24 h after administration of contrast agents. Blood urea nitrogen (BUN) and bilirubin (BILI) had a statistical significance at F < 0.05 in values measured after 15 min, and statistical significance at p < 0.001 were obtained for aspartate aminotransferase (AST) and BILI. Statistical significance at F < 0.05 for the parameters measured after 60 min were BILI and AST, and statistical significance of values at p < 0.001 was obtained for the same parameters. Within 48 h of substance administration, the values of the tested parameters came back within physiological limits. Administration of contrast substance in double dose did not produce changes in pulse, body temperature and respiratory rate.

Key words: Non-ionic contrast medium, dog, blood chemical analysis, administration, intravenous.

INTRODUCTION

Adverse reactions to contrast agents range from a mild inconvenience, such as itching associated with hives, to a life-threatening emergency. Renal toxicity is a well-known adverse reaction associated with the use of intravenous contrast material. Other forms of adverse reactions include delayed allergic reactions, anaphylactic reactions, and local tissue damage (Thomas and Maddox, 2002). Complications from radiographic contrast media depend on variety of factors, including the route of administration, chemical composition of the substance and the patient’s underlying condition. The mechanical effect of needle placement is also a consideration (Paithanapagare et al., 2008). The selection of contrast medium has a crucial role in radio diagnostic examination. The ideal contrast preparation should be minimal neurotoxic, should be pharmacologically inert, miscible with cerebrospinal fluid and radio opaque at an isotonic concentration (Widmer and Blevins, 1991). A number of other studies consistently reported a lower incidence of contrast-induced nephropathy where non-ionic, iso-osmolar contrast media were used, regardless of the route of N-acetylcysteine administration (Baker et al., 2003; Azmus et al., 2005; Ye et al., 2012). The association of such a contrast medium with reduced contrast-induced nephropathy incidence was well

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demonstrated (Soehardy, 2004; Hernandez et al., 2009; Ye et al., 2012).

In our study, we worked with two contrast substances: Optiray 350 (producer Tyco Healthcare Canada) and Ultravist 370 (producer Schering AG - Germany), which are non-ionic contrast substances with a low osmolality. The osmolality of a particular contrast agent is determined by the number of osmotically active particles formed when it is dissolved in solution. Ionic agents dissociate into ions when dissolved in water and contain an iodinated benzene ring. As a result, ionic agents have a higher osmolality than blood. Non-ionic agents do not dissociate into separate particles when dissolved in water; their osmolality is therefore one half that of ionic agents (Thomas and Maddox, 2002). It is concluded that unlike ionic contrast media, non-ionic contrast agents appear to have less inhibitory effects on blood clot formation and thus, it might be associated with a higher risk of thromboembolic complications during coronary angiography (Hwang et al., 1989).

High-osmolar ionic contrast media are more cytotoxic than low-osmolar non-ionic contrast media to gallbladder epithelial cells (Ju et al., 2002). A low-osmolar, ionic contrast medium, has a greater anticoagulant effect than a low-osmolar, non-ionic contrast agent (Kurisu and Tada, 1992). Some studies demonstrated that a higher incidence of contrast-induced nephropathy occurred most often in subjects that have used a low-osmolality ionic contrast medium (for example, iomeprol) (Ye et al., 2012).

The goal of our study was to track the effects of biochemical and hematological changes and modification of physiologic function when double doses of non-ionic contrast substance were administrated intravenously (iv) in anesthetized dogs.

MATERIALS AND METHODS

The study was conducted on a total of 30 adult dogs of different breeds and sexes and an average weight of 20 kg. The animals were divided into two lots of 15 individuals. The study monitored the biochemical and hematological changes induced by administration of a double dose of non-ionic contrast substance (Optiray 350 - group I, Ultravist 370 - group II), with the normal dose administered to dogs for the two substances studied being between 3 to 3.5 ml/kg. We also followed the influence of the dose on the major physiological functions and observed if this dose was life-threatening. Before substance administration, patients underwent 24 h food diets, water was administered ad libitum. Each patient was examined clinically; the non-ionic substance dose was 7.0 ml/kg intravenously. Before administration, patients were subjected to neuroleptanalgesia with xylazine and ketamine (xylazine 1 to 2 mg/kg im bw; Ketamine 10% 0.06 to 1 ml/kg im bw) (products manufactured by Animal Health GMP, Netherlands). We also monitored heart rate, body temperature and respiratory rate after 10 min, 1 and 24 h. Blood samples were collected and analyzed after 15, 60 min and 24 h after substance administration. Non-ionic contrast substances used were administered intravenously in bolus, in the cephalic vein. The biochemical and hematological parameter measurement was performed with the help of the Clinical biochemistry analyzer by Diatron LTD, USA.

Statistical analysis

The changed parameter values in the two lots, following administration of contrast substance were statistically calculated using the mean and standard deviation. To check the variance equality of the two groups, the F test was applied for each parameter change. The Student t-test was used for comparison between the mean changed parameters in the two substances studied. Statistical processing was performed with Microsoft Excel 2010 software (Microsoft).

RESULTS

The results obtained from biochemical analysis of the 15 cases in lot I to which Optiray 350 was given were subjected to statistical calculation, using the mean and standard deviation. After 15 min, after administration of Optiray 350 in lot I, increases in alkaline phosphatase (ALP) 152 ± 3 IU/L, normal value ranging from 12 to 121 IU/L, gamma glutamyl transferase (GGT) 14 ± 1, 65 IU/L values normally ranging from 2 to 10 IU/L, increases in blood urea nitrogen (BUN) 41 ± 3.87 mg/dl normal value ranging from 8 to 30 mg/dl, increases in aspartate aminotransferase (AST) 81 ± 4.97 IU/L value normally ranging between 16 to 54 IU/L and elevated total bilirubin (BIL) 0.76 ± 0.042 mg/dl normal value ranging from 0.1 to 0.3 mg/dl [reference values after Vaden et al. (2009)], were found. The remaining biochemical parameters did not change. Also, there were no reported changes in either the serum protein electrophoresis; values obtained were within normal limits. At the hematological exam, the values obtained were within normal limits except mean corpuscular hemoglobin concentration (MCHC), this parameter showed a slight increase up to 1.5 g/dl from the maximum allowed value (normal value 32 to 36 g/dl).

After 60 min from the administration of Optiray 350, increases in ALP 162 ± 4.23 IU/L, GGT 13 ± 2.24 IU/L, BUN 36 ± 4.47 mg/dl, AST 76 ± 9.65 IU/L and increases in BIL 0.73 ± 0.080 mg/dl, appeared. Regarding the hematological parameter values, in 60 min after administration of Optiray 350, a slightly higher number of red blood cells (RBC) with an average of 0.07 units (normal value 5.8 to 8.5 10^12/l) and an increase in hemoglobin (HGB - normal value 14 to 19.1 g/dl) and mean corpuscular hemoglobin concentration (MCHC - normal value 32 to 36 g/dl) with an average of 0.5 units to the maximum permitted levels, were identified.

In 48 h from Optiray 350 administration, both the mean of the biochemical parameters as well as the mean of the hematological parameters was within the reference interval. In group II, after receiving a double dose of the
non-ionic contrast substance Ultravist 370, after 15 min, ALP 154 ± 3.76 IU/L, GGT 13 ± 2.51 IU/L, BUN 30 ± 2.27 mg/dl, AST 92 ± 4.17 IU/L and 0.68 ± 0.07 BILI mg/dl were increased. At 60 min after a double dose of Ultravist 370, ALP 161 ± 3.63 IU/L, GGT 12 ± 1.46 IU/L, BUN 37 ± 3.48 mg/dl, AST 62 ± 3.59 IU/L and BILI 0.68 ± 0.04 mg/dl were still elevated.

Hematological parameters showed no significant increase, after 15 min we observed an increase in MCHC with an average of 0.99 units to the maximum, and after 60 min after a double dose of Ultravist 370, we noticed slight increases of RBC, with an average of 0.1 units, and of HGB and MCHC with an average of 0.8 units. Values obtained in serum protein electrophoresis showed no changes.

Analyses carried out 48 h after dosing a double dose of Ultravist 370 showed no significant changes, the average values of measured parameters returned to normal limits. To check the variance equality of the two lots, the F test was applied for each parameter change. After statistical processing, we applied the Student t-test, comparing the equality of average values obtained between the two lots. The t-test statistical significance was p < 0.05. Among the parameters measured at 15 min, BUN and BILI had a statistical significance at F < 0.05, and statistical significance at p < 0.001 was obtained for AST and BILI. Among the parameters measured at 60 min, statistical significance (F < 0.05) was found for AST and BILI, and statistical significance at p < 0.001 was determined for AST and BILI. When it came to the values measured after 24 h, F < 0.05 was obtained for BILI, and statistical significance at p < 0.05 was obtained for PAL, at p < 0.001 for BUN and p < 0.01 for AST.

**DISCUSSION**

After the study, in 23% (7 patients) we found mild feelings of discomfort, pain during administration, skin reactions such as hives or erythema and vomiting, symptoms that disappeared a few hours after administration, not being life threatening. Biochemical changes were reported both at 15 and 60 min after administration of contrast substance in both groups studied. Determined biochemical changes were found only in ALP, GGT, BUN, AST, BILI, showing moderately increased values. Rising of total serum ALP is determined by component isoenzymes. The most common isoenzymes are the liver isoenzymes which is increased in cholestasis, but without modification values in cellular damage or necrosis, bone isoenzymes with high values in bone remodeling and corticosteroid-induced isoenzymes produced in liver being specific only for dogs.

Gamma-glutamyltransferase is an enzyme found in different type of tissues, especially in kidney, pancreas and intestinal mucosa, but has an important role in hepatic diseases. GGT value is high in cholestasis or biliary necrosis, the mechanism of increase GGT serum value in this affection is not well known, but could be due to increase GGT production or release of GGT from cells membranes.

Blood urea nitrogen is the final product of protein metabolism and is produced in the liver through urea cycle. BUN is tested for modification of renal function and is also a marker of liver function or liver insufficiency. High level of AST most commonly indicates either hepatocellular or muscle injury. Because red blood cells contain also AST, therefore high level of AST could be found in intravascular hemolytic disease. Serum bilirubin may be increased in prehepatic icterus, hepatic icterus or in posthepatic icterus.

Statistical calculation for AST and BILI values obtained 15 and 60 min after administration of contrast substance (p < 0.001) shows the direct link between changes in parameters and the contrast substance. In 24 h, the statistical significance at p < 0.5 was shown for ALP, at p < 0.001 for BUN and p < 0.01 for AST. Moderate increase of ALP and GGT correlated with a slight increase of BILI were indicating a possible cholestasis and biliary reactivity due to the metabolism of the contrast substances, the values exceeded the maximum limit by only a couple of units. Increased urea correlated with increased GGT appeared due to the effect of the active substance on the kidney caused by mild intravascular hemolysis, mild cholestasis and a possible acute tubular reaction. Increased urea did not produce morphological changes on the kidney, biochemical examination conducted one week after administration of contrast showed normal levels of urea, and radiological and ultrasound examination did not show changes in the renal parenchyma.

Administration of contrast substance in bolus correlated with the low osmolarity of the substances can cause a possible intravascular hemolysis which can lead to increased AST and MCHC. Relative increase in hemoglobin and red blood cells indicated a possible splenic contraction reaction to the contrast substance. The electrophoresis of serum proteins did not change from the normal values for both substances studied.

Non-ionic contrast substances, when properly administered, are not painful, the optimal dose of Optiray 350 is 3.5 ml/kg and the optimal dose of Ultravist 370 is 3 ml/kg. Double doses of contrast substance increases metabolism time, therefore, in radiological examinations, the image quality does not change, and there are no life-threatening effects. Biochemical and hematological changes induced by administered contrast substances returned to physiological values after 48 h. The products tested had excellent neural tolerance and a minimal influence on the cardio-respiratory system.
Conclusion

The effect of non-ionic contrast substances on the liver and kidneys were minimal, no morphological or functional changes were noticed in a week after substance administration.

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REFERENCES


Assessment of frequently accessible homeopathic mother tinctures for their pharmacopoeal specifications in Pakistan

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The homeopathic system of medicine has attained ample attentiveness being one of the premium systems of cure accessible to mankind, and lingers to hold the awareness of remedial publications and the correspondence at the same time. The foremost superlative of cure is swift, undeviating refurbishment of health or inhibitive of the disease in its entire scope, most trustworthy and non detrimental way. The probable means of their accomplishments seem conflicting by way of conformist procedural deliberation and the investigation corroboration for which leftovers are arguable. Mother tinctures are liquid preparation ensuing from the extraction of an appropriate source that is, animal substance material with alcohol or water mixture within a specific ratio. Adverse events taking place during homeopathic treatment are seldom accredited to the homeopathic medicine itself. Nevertheless, sanctuary appraisal should also deem possible impurities of the source material or contagion and failures of good manufacturing practice. This is the first study conducted in Pakistan to appraise the physical and chemical parameters of five frequently accessible mother tinctures that is, Aconitum napellus, Arnica montana, Bryonia alba, Atropa belladonna and Matricaria chamomilla, and manufactured by five confined leading companies by employing customary methodology for the following specifications that is, alcohol contents, weight/ml, specific gravity and pH. Most of the parameters of mother tinctures comply with German Homoeopathic Pharmacopoeia and manufacturers own specifications. There is dire need to enact law to control the sale, manufacture, storage and export of complementary and alternative medicines at earliest possible time in true letter and spirit, along with the adaptation of good manufacturing practice (GMP), quality control (QC) and quality assurance (QA) guideline.

Key words: Mother tinctures, Aconitum napellus, Arnica montana, Bryonia alba, Atropa belladonna, Matricaria chamomilla, pharmacopoeal specifications.

INTRODUCTION

Medicinal plants have been manipulated over a period of time in the cure of ailments due to the pharmacological prospective and organic commotion of the compounds inside the plant (Ceśla and Waksmundzka-Hojnos, 2009), and also represents a foundation of unprocessed materials for both contemporary and conventional types of medicine that is, Homoeopathy, Unani, Chinese and Ayurvedic medicine (Bandaranayake, 2006). There has been a substantial awareness worldwide in Traditional medicine/ Complementary and Alternative Medicine (TCAM) particularly in herbal products over the last few decades

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The homeopathic system of medicine is one of the premium systems of cure accessible to mankind. The premier superlative of cure is swift, moderate and undeviating refurbishment of health or confiscation and inhibitive of the disease in its entire scope, in the unsnerving, most reliable and most not detrimental way, on easily ample principles (www.nchpakistan.com). The National regulatory scaffold and the place of homeopathy within the health care system diverge from country to country, but the use of homeopathic medicines, mostly as non prescription medicines, is emerging in numerous parts of the world. The literal size of the homeopathic medicines market in cost-effective term is not well known but sales data divulge that homeopathic medicines represent a noteworthy part of medical economics (WHO, 2009).

A mother tincture is a liquid preparation ensuing from the extraction of a suitable source, namely plant or animal substance material with alcohol or water mixture within a specific ratio (Banerjee, 2002). They have widespread restorative account and as the prominence of wellbeing is fetching a wide-reaching tendency, homeopathic provisions are becoming progressively more accepted. Nevertheless (Pande and Pathak, 2006). Homeopathic mother tinctures, rarely and in amalgamations, contains a lots of figures of compounds in composite matrices in which no solitary vigorous component is accountable for the over all effectiveness. This creates a confrontation in establishing excellent run principles for unprocessed materials and equivalence of refined herbal drugs, ensuing in anecdotal values for these provisions internationally (Chitlange, 2008). Its standardization is usually done by assorted physical, chemical and chromatographic specifications.

As the international costs of mother tinctures have begun to escalate due to assorted factors, the use of local manufacturers has become an imperative source option. A need therefore has arisen to frequently assess whether there is a difference in quality between locally and internationally feigned mother tinctures and whether the quality of the local and international manufactured homeopathic mother tinctures comply with the standard stipulated by the Homoeopathic Pharmacopoeia (Lee et al., 2007).

The high impending inconsistency of chemical composition of the plant material implicated in the construct of homeopathic mother tinctures renders both their quality control and assurance a noteworthy confront (Pande and Pathak, 2006). Homeopathic medicines or their stocks/mother tinctures are prepared from natural or synthetic sources that are referenced in pharmacopoeial monographs or other renowned documents. Numerous factors have been reported to manipulate the excellence of homeopathic mother tinctures, and countless specifications must be met when assessing the worth of these products (Bandaranayake, 2006).

Not considering imponderabilia, the source materials for homeopathic medicines may consist of plant material such as: roots, stems, leaves, flowers, bark, pollen, lichen, moss, ferns and algae; microorganisms such as: fungi, bacteria, viruses and plant parasites; animal materials such as: whole animals, animal organs, tissues, secretion, cell lines, toxins, nosodes, blood products; human materials such as: tissues, secretion, cell lines; and endogenous molecules such as hormones, minerals and chemicals. Furthermore, the quantity of starting material present in homeopathic medicines may depend on the method of preparation.

Safety issues may crop up if these differences in method of preparation are abandoned. For example, an assessment of the "identically" entitled pharmacopoeial monographs on Aconitum napellus in different pharmacopoeias that is, the Pharmacopoeia française (Phf), the German Homeopathic Pharmacopoeia (GHP), the Homoeopathic Pharmacopoeia of the United States (HPUS) and the Homoeopathic Pharmacopoeia of India (HPI) reveals substantial differences (Table 1). Aconitum napellus 1X = 1DH prepared according to the German Homeopathic Pharmacopoeia is closer to A. napellus mother tincture than to the 1X = 1DH, both prepared according to the Pharmacopoeia française (WHO, 2009).

Diverse customs in the utilization of homeopathic and anthroposophic analysis and altering levels of proficiency along with the dogmatic powers inside the European unification have executed in altering principles of appraisal (Schultz et al., 2011). Quality assurance for herbal starting materials is based on the knowledge about their origins, as well as their pharmacognosic identification and assessment. The studies had shown that the thin layered chromatography (TLC) fingerprints of different mother tincture batches may show wide variation. This raises the question of whether additional quality characteristics should be considered in order to ensure a constant and uniform level of quality. For this purpose, it seems particularly appropriate to use the concept of marker substances, as in the field of Herbal medicinal products if this concept were to be applied to mother tinctures. The fortitude of a specific marker substance, or group of marker substance, would have to be added to the current range of quality characteristics. In this context, however, it is important to note that the current knowledge about characteristic constituents of plants has for the most part been gained from studies of dried plant materials (Hager et al., 2006).

Adverse events taking place during homeopathic treatment are rarely accredited to the homeopathic medicine itself. However, safety assessment should also consider possible impurities of the source material or contamination and failures of good manufacturing practice. Furthermore, because many homeopathic medicines can be
medicines can be purchased as non-prescription medicines in community pharmacies and health stores, without consultation efforts with a healthcare provider, it has become increasingly important to provide sufficient and accessible information on such medicines. Although homeopathic medicines are generally assumed to be benign, the level of authorization, appropriate labeling and quality assurance should take into consideration its extensive use, also within vulnerable populations such as the elderly, pregnant women and children.

In recent years, there have been a number of calls on WHO to support efforts to regulate the safety of homeopathic medicines (WHO, 2009). Throughout the precedent few decades, the manipulation of homeopathic preparations and herbal medicines in the industrial world has turn into a trendy and exceedingly demanded form of therapeutic management, which has been expedited by fewer rigorous systems than other medications (WHO, 2009c). Even though the majority of trustworthy manufacturers of homeopathic mother tinctures point toward the charisma of vigorous components on the labels of unvarying products, methodical methods have mottled extensively (Gower, 2009).

The current study was conducted to appraise the physical and chemical parameters of five frequently accessible used mother tinctures that is, Aconitum napellus, Arnica montana, Bryonia alba, Belladonna and Chamomila, prepared by five local homeopathic pharmaceutical companies by employing standard methodology for following parameters that is, alcohol contents, weight/ml, specific gravity and pH. All of these mother tinctures are commonly used in different dilutions throughout the country for the cure of different diseases.

MATERIALS AND METHODS

Sample collection and physico-chemical properties assessment

Twenty five samples of mother tinctures were collected from various localities of the country that is, Islamabad, Rawalpindi, Lahore and the local market during August, 2011. All samples were stored at room temperature by employing WHO guidelines on good agricultural and collection practice (GACP) and FCP (Field collection practices) (WHO, 2003). Mother tinctures of conitum A. napellus, A. montana, B. alba, Belladonna and Chamomila manufactured by four various homeopathic companies were purchased from open market and were analyzed for the following parameters by employing standard methodology (British Pharmacopoeia (BP), 1968).

1. Alcohol contents;
2. Weight/ml;
3. Specific gravity;
4. pH.

There are three methods in BP (1968) and we had employed all three methods according to the need and requirement of parameters.

Method-I

Twenty five (25) ml of the preparation was measured in a graduated flask at 20°C, transferred to a flask of 500 to 800 ml capacity, the graduated flask was washed with 100 to 150 ml of water, and the washings added to the contents of the larger flask together with a little pumice powder. The flask was connected to a condenser by means of a suitable still-head, and distilled for at least 90 ml, into a 100 ml graduated flask. The distillate was brought to 20.0°C and diluted to 100 ml with water at the same temperature. The specific gravity was determined at 20.0°C and the refractive index of the solution at 20.0°C. And if by reference to the ethyl alcohol (Quadruple bulk) table, page 1280, the refractive index does not differ by more than 0.00007 (equivalence of 0.2 on the immersion refractive index does not differ by more than that corresponding to the specific gravity, the percentage of ethyl alcohol corresponding to the specific gravity is read off). If the refractive index differs by more than 0.00007 (equivalence of 0.2 on the immersion refractive index scale), 75 ml of the distillate is treated with powdered sodium chloride and light petroleum (boiling-range, 40 to 60°), as in Method II, distilled to about 70 ml, and the distillate to 75 ml. If the refractive index still does not correspond with the specific gravity, the distillate contains some impurity, and the specific gravity does not indicate the true proportion of ethyl alcohol. When the distillate contains steam-volatile substances other than alcohol (it will then usually be turbid or contain oily drops), Method III is applied. When steam volatile-acids are present, the solution is made alkaline with N/I sodium hydroxide, using solid phenolphthalein as indicator before the final distillation.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pharmacopoeia Française (PhF)</th>
<th>German Homeopathic Pharmacopoeia (GHP)</th>
<th>Homoeopathic pharmacopoeia of the United States (HPUS)</th>
<th>Homoeopathic pharmacopoeia of India (HPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid content expressed in aconitine in the mother tincture</td>
<td>0.02–0.05%</td>
<td>0.08–0.16%</td>
<td>0.025–0.075%</td>
<td>Not described (approx. 0.03%)</td>
</tr>
<tr>
<td>Ratio of mother tincture to diluents for obtention of 1X = 1DH</td>
<td>1:9</td>
<td>1:4</td>
<td>1:1</td>
<td>Mother tincture = 1X</td>
</tr>
<tr>
<td>Percentage of the mother tincture in the 1DH dilution</td>
<td>10%</td>
<td>20%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Content expressed in aconitine in 1X = 1DH</td>
<td>0.002–0.005%</td>
<td>0.016–0.032%</td>
<td>0.025–0.075%</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Method II
Twenty five (25) ml of the preparation was measured in a graduated flask at 20°C, transferred to a separator, the graduated flask washed with about 100 ml of water, the washings added to the contents of the separator with sufficient powder (boiling-range, 40 to 60°), and shaken vigorously for two to three minutes. The mixture was allowed to stand from 15 to 30 min and run power layer run into a distillation flask. The light petroleum was washed in the separator by shaking vigorously with about 25 ml of a saturated solution of sodium chloride, allowed to stand, and the washed liquor run into the first brine solution. Where the alcohol limits table directs a double separation, the brine solution was run into a second separator and shaken further with 100 ml of light petroleum (boiling-range, 40 to 60°) before transferring to the distillation flask. This is the second quantity of light petroleum with the washed liquor from the first washing. The mixed solutions were made alkaline with N/1 sodium hydroxide using solid phenolphthalein as indicator, a little pumice powder was added, and together with 100 ml of water the solution was distilled 90 ml. The amount of ethyl alcohol was determined by Method I. The distillate was brought to 20.0°C and diluted to 100 ml with water at the same temperature.

The specific gravity was determined at 20.0°C, and the refractive index of the solution at 20.0°C, and if, by reference to the ethyl alcohol (Quadruple Bulk) table, page 1280, the refractive index does not differ by more than 0.00007 (equivalence of 0.2 on the immersion refractive index does not differ by more than corresponding to the specific gravity) the percentage of ethyl alcohol corresponding to the specific gravity is read off. If the refractive index differs by more than 0.00007 (equivalence of 0.2 on the immersion refractometer scale), 75 ml of the distillate is washed with powdered sodium chloride and light petroleum (boiling-range, 40 to 60°) before being treated with powdered sodium chloride and light petroleum (boiling range, 40 to 60°): as in Method II, about 70 ml was distilled and diluted to 75 ml.

If the refractive index still does not correspond with the specific gravity, the distillate contained some impurity, and the specific gravity does not indicate the true proportion of ethyl alcohol. When the distillate contains steam-volatile substances other than alcohol (it will then usually be turbid or contain oily drops), Method III is used. When steam volatile-volatile acids are present, the solution was made alkaline with N/1 sodium hydroxide, using solid phenolphthalein as indicator before the final distillation.

Method III
Twenty five (25) ml of the preparation was measured in a graduated flask at 20°C, transferred to a flask of 500 to 800 ml capacity, washed with 100 to 150 ml of water with the washings added to the contents of the larger flask, and a little pumice powder finally added. The flask was connected to a condenser by means of a suitable still-head, and distilled to about 100 ml. The solution was transferred to a separator, and the amount of ethyl alcohol determined by Method II. Sufficient powdered sodium chloride was added and allowed to stand. The wash liquor was finally run into the first brine solution. Where the alcohol limits table directs a double separation, the brine solution was run into a second separator and shaken further with 100 ml of light petroleum (boiling-range, 40 to 60°) before transferring to the distillation flask. This second quantity of light petroleum was washed with the washing liquor from the first washing. The mixed solutions were made alkaline with N/1 sodium hydroxide, using solid phenolphthalein as indicator, with pumice powder and 100 ml water added and distilled to 90 ml. The amount of ethyl alcohol was determined by Method I.

The distillate was brought to 20.0°C and diluted to 100 ml with water at the same temperature. The specific gravity at 20.0°C was determined, and the refractive index of the solution at 20.0°C also, and if by reference to the ethyl alcohol (Quadruple Bulk) table, page 1280, the refractive index does not differ by more than 0.00007 (an equivalence of 0.2 on the immersion refractive index does not differ by more than corresponding to the specific gravity), the percentage of ethyl alcohol corresponding to the specific gravity is read off. If the refractive index differs by more than 0.00007 (equivalence of 0.2 on the immersion refractometer scale), 75 ml of the distillate is treated with powdered sodium chloride and light petroleum (boiling range, 40 to 60°): as in Method II, about 70 ml was distilled and diluted to 75 ml.

If the refractive index still does not correspond with the specific gravity, the distillate contained some impurity, and the specific gravity does not indicate the true proportion of ethyl alcohol. When the distillate contains steam-volatile substances other than alcohol (it will then usually be turbid or contain oily drops), Method III is used. When steam volatile-volatile acids are present, the solution was made alkaline with N/1 sodium hydroxide, using solid phenolphthalein as indicator before the final distillation.

RESULTS
A total of 25 samples of commonly used mother tinctures of A. napellus, A. montana, B. alba, Belladonna, Chamomila manufactured by five different companies were unrefined from diverse places of the country that is, Islamabad, Rawalpindi, Lahore and physicochemical specifications that is, alcohol contents, weight/ml, specific gravity, pH were determined and analyzed statistically.

Table 2 shows that alcohol content of A. napellus of five mother tinctures varies from 59.01 to 64.55% (Range 61 to 65%, standard deviation = 2.40), weight/ml varies from 0.859 to 0.884 g/ml (range = 0.890 to 0.925, SD = 0.00956). Specific gravity varies from 0.903 to 0.929 (range is 0.930 to 0.950 = according to GHP and SD = 0.00998) and pH varies from 5.06 to 6.099 (range = 5.0 to 7.0 and SD = 0.41367).

All ranges given in Table 1 are given by manufacturers except specific gravity which was not given by manufacturers and given in German Homeopathic Pharmacopoeia. Table 3 shows results of A. montana whose alcohol contents varies from 53.71 to 62.73% (range 57 to 61%) except of Rax Laboratories whose result is 83.11% with the range of 88 to 92%. Weight/ml varies from 0.788 to 0.884 (Range = 0.900 to 0.925, SD = 0.03857); specific gravity varies from 0.828 to 0.929 (range = 0.833 to 0.945 GHP, SD = 0.04108), pH varies from 5.060 to 6.099 (Range = 5.0 to 7.0, SD = 0.41367). Range of specific gravity is taken from GHP. Table 4 shows results of B. Alba whose alcohol contents varies from 51.94 to 57.28% (range 57 to 61%) except of BM Pvt. Ltd whose result is 55.48%, with the range of 60 to 70%.

Weight/ml varies from 0.870 to 0.892 (Range = 0.883 to 0.940, SD = 0.00953), specific gravity varies from 0.914 to 0.920 (range = 0.940 to 0.960 GHP, SD = 0.00249), pH varies from 5.932 to 6.939 (range = 5.5 to 8.3) except of BM Pvt. Ltd whose result is 5.959 with the range of 7.9 to 8.3. Table 5 shows results of Belladonna whose alcohol content varies from 36.63 to 53.71% with different ranges given by all companies. Weight/ml varies from
Table 2. Name of the drug (A. napellus).

<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Alcohol content (%)</th>
<th>Weight/ml (g/ml)</th>
<th>Specific gravity</th>
<th>pH</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
</tr>
<tr>
<td>Aconitum napellus</td>
<td>59.01</td>
<td>61</td>
<td>0.8662</td>
<td>0.90</td>
<td>0.910</td>
</tr>
<tr>
<td>Aconitum napellus</td>
<td>64.55</td>
<td>61-65</td>
<td>0.859</td>
<td>-</td>
<td>0.903</td>
</tr>
<tr>
<td>Aconitum napellus</td>
<td>64.55</td>
<td>61-65</td>
<td>0.884</td>
<td>0.890-0.925</td>
<td>0.929</td>
</tr>
<tr>
<td>Aconitum napellus</td>
<td>62.72</td>
<td>63</td>
<td>0.868</td>
<td>-</td>
<td>0.912</td>
</tr>
<tr>
<td>Aconitum napellus</td>
<td>60.90</td>
<td>61-65</td>
<td>0.863</td>
<td>0.896-0.904</td>
<td>0.907</td>
</tr>
</tbody>
</table>

*KL= Kamal Laboratories, Rawalpindi Pakistan; RL= Rax Laboratories, Homeopathic, Lahore Pakistan; BM= BM (private) Limited Lahore Pakistan; WH= Warsan homeopathic Laboratories, Lahore Pakistan; MH = Masood homeopathic Stores & Hospitals, Lahore, Pakistan.

Table 3. Name of the drug (Arnica Montana).

<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Alcohol content (%)</th>
<th>Weight/ml (g/ml)</th>
<th>Specific gravity</th>
<th>pH</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
</tr>
<tr>
<td>Arnica montana</td>
<td>53.71</td>
<td>60</td>
<td>0.86</td>
<td>0.80</td>
<td>0.913</td>
</tr>
<tr>
<td>Arnica montana</td>
<td>83.11</td>
<td>88-92</td>
<td>0.788</td>
<td>-</td>
<td>0.828</td>
</tr>
<tr>
<td>Arnica montana</td>
<td>55.48</td>
<td>57-61</td>
<td>0.884</td>
<td>0.900-0.925</td>
<td>0.929</td>
</tr>
<tr>
<td>Arnica montana</td>
<td>60.91</td>
<td>57-61</td>
<td>0.869</td>
<td>0.900-0.925</td>
<td>0.914</td>
</tr>
<tr>
<td>Arnica montana</td>
<td>62.73</td>
<td>57-61</td>
<td>0.875</td>
<td>0.900-0.925</td>
<td>0.919</td>
</tr>
</tbody>
</table>

*KL= Kamal Laboratories, Rawalpindi Pakistan; RL= Rax Laboratories, Homeopathic, Lahore Pakistan; BM= BM (private) Limited Lahore Pakistan; WH= Warsan homeopathic Laboratories, Lahore Pakistan; MH = Masood homeopathic Stores & Hospitals, Lahore, Pakistan.

Table 4. Name of the drug (Bryonia alba).

<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Alcohol contents (%)</th>
<th>Weight/ml (g/ml)</th>
<th>Specific gravity</th>
<th>pH</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
</tr>
<tr>
<td>Bryonia alba</td>
<td>51.94</td>
<td>60</td>
<td>0.870</td>
<td>0.900</td>
<td>0.914</td>
</tr>
<tr>
<td>Bryonia alba</td>
<td>57.28</td>
<td>57-61</td>
<td>0.870</td>
<td>-</td>
<td>0.914</td>
</tr>
<tr>
<td>Bryonia alba</td>
<td>55.48</td>
<td>60-70</td>
<td>0.875</td>
<td>0.883-0.940</td>
<td>0.920</td>
</tr>
<tr>
<td>Bryonia alba</td>
<td>53.71</td>
<td>57-61</td>
<td>0.892</td>
<td>0.983-0.940</td>
<td>0.916</td>
</tr>
<tr>
<td>Bryonia alba</td>
<td>51.94</td>
<td>57-61</td>
<td>0.870</td>
<td>0.883-0.940</td>
<td>0.917</td>
</tr>
</tbody>
</table>

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Table 5. Name of the drug (Belladonna)

<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Alcohol contents (%)</th>
<th>Weight/ml (g/ml)</th>
<th>Specific gravity</th>
<th>pH</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
<td>Limit</td>
<td>Limit (GHP)</td>
</tr>
<tr>
<td>Belladonna</td>
<td>36.63</td>
<td>45</td>
<td>0.789</td>
<td>0.928</td>
<td>0.829</td>
</tr>
<tr>
<td>Belladonna</td>
<td>45.02</td>
<td>47-51</td>
<td>0.887</td>
<td>-</td>
<td>0.932</td>
</tr>
<tr>
<td>Belladonna</td>
<td>53.71</td>
<td>45-55</td>
<td>0.911</td>
<td>0.900-0.960</td>
<td>0.957</td>
</tr>
<tr>
<td>Belladonna</td>
<td>43.32</td>
<td>41-45</td>
<td>0.910</td>
<td>0.926-0.948</td>
<td>0.956</td>
</tr>
<tr>
<td>Belladonna</td>
<td>45.02</td>
<td>41-45</td>
<td>0.904</td>
<td>0.926-0.948</td>
<td>0.950</td>
</tr>
</tbody>
</table>

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Table 6. Name of the drug (Chamomilla).

<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Alcohol contents (%)</th>
<th>Weight/ml (g/ml)</th>
<th>Specific gravity</th>
<th>pH</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed value</td>
<td>Limit</td>
<td>Observed value</td>
<td>Limit</td>
<td>Limit (GHP)</td>
</tr>
<tr>
<td>Chamomilla</td>
<td>39.95</td>
<td>45</td>
<td>0.894</td>
<td>0.924</td>
<td>0.941</td>
</tr>
<tr>
<td>Chamomilla</td>
<td>62.72</td>
<td>67-71</td>
<td>0.859</td>
<td>-</td>
<td>0.902</td>
</tr>
<tr>
<td>Chamomilla</td>
<td>45.02</td>
<td>45-55</td>
<td>0.894</td>
<td>0.920-0.960</td>
<td>0.940</td>
</tr>
<tr>
<td>Chamomilla</td>
<td>46.73</td>
<td>47-51</td>
<td>0.970</td>
<td>0.910-0.940</td>
<td>0.953</td>
</tr>
<tr>
<td>Chamomilla</td>
<td>51.94</td>
<td>47-51</td>
<td>0.891</td>
<td>0.910-0.940</td>
<td>0.937</td>
</tr>
</tbody>
</table>

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0.789 to 0.911 (range = 0.900 to 0.960, SD = 0.05188), specific gravity varies from 0.829 to 0.957 (range = 0.932 to 0.947 GHP, SD = 0.05449), pH varies from 6.130 to 6.492 (different ranges given by all manufacturers).

Table 6 shows results of Chamomilla whose alcohol content varies from 39.95 to 62.72% with different ranges given by all companies. Weight/ml varies from 0.859 to 0.970 (range =0.910 to 0.960) except of BM Pvt. Ltd whose result is 0.894 with the range of 0.920 to 0.960, specific gravity varies from 0.902 to 0.953 (range = 0.900 to 0.920 GHP, SD = 0.01922) and pH varies from 5.339 to 6.275 (range = 5.5 to 6.5).

**DISCUSSION**

Homeopathic medicine continues to catch the attention of medical journals and the media as a well-liked form of complementary medicine (CM) whose anticipated mechanism of action seems irreconcilable with conventional technical thought and the research substantiation which remains contentious. Categorization of the prelude matter is guaranteed by the eminence of homoeopathic mother tinctures, the industrialized procedure and the exploratory distinctiveness construed in the treatise (Biber et al., 2009). Very little data is available on the quality of homoeopathic medicines as there is no law to regulate the complementary and alternative system of medicines (CAM) and is self regulatory. The most frequently
used but famous forms of CAM is homeopathy. Extremely diluted preparations of substances that cause symptoms in healthy individuals are used to kindle remedial reactions in patients who demonstrate analogous symptoms when sick (Jonas and Jacobs, 1996).

Currently, the only quantitative method described in HAB monographs is the fortitude of dry residue, except in those cases where the mother tincture contains toxic compounds. By distinction, the French Pharmacopoeia monographs often include the grit of a marker substance or group determination. Probing for the marker substances in mother tinctures is complicated. Research on plant constituents described in literature was for the most part performed on dried plant materials. These findings cannot be applied to fresh plants in general. This is most likely due to certain factors such as post harvest enzymatic process caused by disruption of the cellular compartmentation (Biber et al., 2009). Our analysis has shown variation in results of all samples of five manufacturers. All samples have their own limits of all parameters even all are from ISO 9001:2008 certified companies.

Generally, the eminence of the elected homoeopathic mother tinctures feigned globally abides by the excellence values deposit by the GHP and GMP. An assortment of samples of the nearby contrived homoeopathic mother tinctures complied with the standards of the GHP and GMP, although others are futile to attain this customary eminence (Scheepmaker, 2010). The quality of source materials and of the excipients used in the manufacture of homoeopathic medicines is imperative. Homeopathic medicines may utilize material from problematic sources, the use of which is constrained in conventional medicine: nosodes comprise dilutions of pathogenic organs or tissues; causative agents such as bacteria, fungi, ova, parasites, virus particles, and yeast; disease products, excretions or secretions. All materials of animal or human origin are at risk of containing pathogenic agents. Homeopathic medicines may be based on toxic source materials from animals or plants, while others, particularly in their fresh form are prone to degradation processes or microbiological contamination (WHO, 2009).

Today, quantitative determinations present the analytical state of knowledge for herbal preparations as well. However, for homoeopathic mother tinctures of plant, it is attributed that the whole preparation is considered to present the active substance and in addition, there is no affiliation between dose and effect. For these reasons, quality cannot be proscribed based on one individual active constituent only. Unswerving quality primarily has to be assured by careful control of the starting material and by constancy of the traditional manufacturing process. Analogous to other herbal preparations it could be possible to select certain markers for quality control of homoeopathic mother tinctures made from plants in mother tinctures containing strong acting or toxic compounds, the contents of these substances have to be checked anyway for reasons of medicinal product safety (Directive, 2001/83/EC).

Plant materials may be tainted with pesticides and heavy metals. The content of toxic constituents in plant materials may vary considerably. Good manufacturing practice (GMP) guidelines covering the manufacturing process, premises, personnel, packaging and labeling apply to homeopathic medicines as well as to conventional pharmaceuticals. Failure to apply GMP may lead to major quality and safety concerns such as misidentification, impurity of starting material, cross-contamination or incidental contamination. The unique characteristics of the manufacturing of homoeopathic medicines have a number of specific implications and demand specially qualified and experienced personnel. These have to handle toxic materials, particularly fresh ones that are prone to degradation processes and microbial contamination; and homeopathic medicines derived from animals or human sources. The properties of homeopathic medicines can be compromised by accidental or intentional contamination of source materials, excipients or diluents, or by the vessel or bottle in which the dilution is made. Because definitions may vary between pharmacopoeias, and because of the wide range of processing techniques and manufacturing methods in the various pharmacopoeias, the final homeopathic products may show marked variability (WHO, 2009).

In Europe, there are two official homeopathic pharmacopoeias regulating production and quality parameters of homeopathic medicinal products based on one years of experience, the German Homoeopathic Pharmacopoeia and the homoeopathic part of the French Pharmacopoeia. The current therapeutic use of homoeopathic medicinal products in Europe is based on the quality stipulated in these pharmacopoeias. Today at the European level, monographs on homoeopathic preparations are integrated step by step into the European pharmacopoeia (Ph. Eur). Replacing National requirements in addition, the European legislative had recently taken up a section on quality of homoeopathic medicinal products of the first time (Directive 2001/83/EC). The dearth of momentous regulations concerning the quality of CAM in South Africa contributes to this challenge (Gqaleni et al., 2007).

The Drug Control and Traditional Medicines Division of the National Institute of Health, Islamabad in collaboration with WHO has organized number of seminars and workshops to educate the stakeholders of traditional medicines sector to adapt and implement current good manufacturing practices, quality control and quality assurance guidelines for herbal medicines and has also developed few booklets as well (Hussain et al., 2011; Shaikh et al., 2009). There is a dire need to implement these recommendations which had emanated from these activities. The present study should be extended to other commonly used homoeopathic medicines which will in turn form the basis of homoeopathic pharmacopoeia.
REFERENCES


Malik et al.          1381


Full Length Research Paper

Antioxidant ability of squid ink polysaccharides as well as their protective effects on deoxyribonucleic acid DNA damage in vitro

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Squid ink polysaccharides (SIPS) were isolated from squid ink, a plentiful and multifunctional marine material, and were demonstrated to have amelioratory effects on cyclophosphamide-induced damage in internal organs of model animals by our previous reports. To further investigate the protective effects of SIPS on chemotherapeutic damage caused by cyclophosphamide, this paper evaluated the bioactivities of the marine polysaccharides with a view to their antioxidant ability and their protective effects on deoxyribonucleic acid (DNA) damage using tests such as hydroxyl radicals, reducing power assay and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals, and pEGFP-N1 plasmid DNA treated with ultra violet (UV) plus H₂O₂. Data revealed that SIPS not only quenched hydroxyl radicals and 1,1-Diphenyl-2-picrylhydrazyl radicals, but showed its strong reducing power and also suppressed oxidative scission on plasmid DNA strand caused by hydroxyl radicals which originated from H₂O₂ shown by UV.

Key words: Squid ink polysaccharides (SIPS), antioxidant ability, deoxyribonucleic acid (DNA) damage

INTRODUCTION

It is well known that reactive oxygen species (ROS) produced in cells may induce oxidative damage to various biomacromolecules in cells, such as polysaccharide, protein, lipid and deoxyribonucleic acid, which results in metabolic and functional disturbance in cells and in turn leads to various pathological changes, for example aging and cancer, two major problems for human originated from ROS induced oxidative stress and deoxyribonucleic acid (DNA) damage (Cerutti, 1994; Wiseman and Halliwell, 1996). Thus quenching ROS in cells must be an effective way to prevent ROS-mediated oxidative damage induced by cellular metabolism and exogenous agents, especially some oxidative drugs such as cyclophosphamide, a most commonly used chemotherapeutic agent for cancer, which is also used in the treatment of some connective tissue and autoimmune diseases, minimal lesion glomerulonephritis, and for the control of organ rejection after transplantation (Emadi et al., 2009). Owing to the strong negative effects of cyclophosphamide-induced ROS on normal tissues except for its positive roles on tumor tissues (Emadi et al., 2009), resultant insufficient dose of cyclophosphamide results in an unfavourable therapeutic effect on cancer. So, development and application of natural antioxidants would be helpful to impair oxidative damage induced by cyclophosphamide and in turn improve the therapeutic effect of the chemotherapeutic agent on tumor.

It was reported that squid ink polysaccharides (SIPS) is a type of glycosaminoglycon with unique structure, [3GlcAβ1-4(GalNAcc1-3)-Fuca1], (Takaya et al., 1996; Chen et al., 2008) and has been unveiled in our previous investigation (Liu et al., 2012) that the bioactive polysaccharides could increase antioxidant ability of some tissues including liver, heart, lung and kidney of model...
animals treated with cyclophosphamide, which implied that SIPS protected normal tissues of model animals from damage induced by chemotherapeutic drugs. Although the antagonism of SIPS on cyclophosphamide-induced oxidative stress has been discovered in vivo, the direct antioxidation of the natural marine polysaccharides is still unknown to us. In the present paper, in vitro investigation was carried out on the free radical scavenging ability of SIPS on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals and hydroxyl radicals, as well as its protective effects on DNA damage in a systematic way. The results should be beneficial to further elaborate the protective effects of SIPS on chemotherapeutic damage induced by cyclophosphamide, and improving the development of natural marine cytoprotectant as well as its clinical application for treatment on tumor.

MATERIALS AND METHODS

General

GeneJET™ polymerase chain reaction (PCR) Purification Kit was purchased from Fermentas, Ontario, Canada. Biowest Regular Agarose G-10 was produced in Spain. Papain and DPPH were purchased from Sigma. Red prussiate of potash was produced by Shanghai Lingfeng Chemical Co. Ltd. FeCl₃ and EDTA-Na₂-Fe were from Guangzhou Chemical Co. Rompyrogallol red was produced by Tokyo Chemical Industry Co. Ltd. ChemiDoc™ XRS+ System was purchased from Bio-Rad Lab. Inc. USA.

Preparation of SIPS

Fresh squid ink sacs collected from local market of aquatic products were stored at -28°C before use. SIPS was prepared via this procedure: ink harvested from dissected sacs thawed at 4°C was suspended into phosphate buffer (pH 6.7), and was then well grinded. After ultrasonic treatment, the ink solution was stored at 4°C for 24 h and was then centrifuged at speed of 14,000 g for 1 h at 4°C.

The supernatant was digested with 1% papain in phosphate buffer (pH 6.7) at 60°C for 24 h, mixed with 1/4 volume liquid mixture of chloroform and n-butanol (v/v, 4/1) followed by stirring for 30 min on a magnetic stirrer. After centrifugation at 5,000 g for 15 min, the supernatant was digested with papain again, the digestion process was performed twice. SIPS in the resultant supernatant was precipitated with four volumes of absolute alcohol and was dried at 50°C.

Reducing power assay

The mixture containing SIPS solution (scheduled concentrations, 2 ml), phosphate buffer saline (PBS) (pH 6.8, 2.5 ml/L, 2 ml) and K₃Fe(CN)₆ solution (1%, 2 ml) was heated in water bath at 50°C for 20 min, cooled with ice water and then mixed with trichloroacetic acid (10%, 2 ml) followed by centrifugation at 3,000 g for 10 min. Distilled water (2 ml) and FeCl₃ solution (0.1%, 0.4 ml) were in turn added into 2 ml of supernatant. After 10 min, optical density (OD) values at wavelength of 700 nm were measured (Shimada et al., 1992).

Detection of scavenging ability on hydroxyl radicals

SIPS (1 ml) and H₂O₂ (2%, 1 ml) were in turn added into Tris-HCl buffer (0.15 mol/L, pH 8.2, 3 ml) containing bromopyrogallol red (1 mmol/L, 1 ml) and EDTA-Na₂-Fe (1 mmol/L, 1 ml). Distilled water was used instead of SIPS in blank tube, and instead of SIPS and EDTA-Na₂-Fe in control tube. After 30 min, OD values at wavelength of 520 nm were measured. Scavenging ratio was calculated as follows:

Scavenging ratio = (OD_BT - OD_ST) / OD_BT × 100%

where BT = blank tubes, ST = sample tubes

Detection of quenching ability on DPPH radicals

The mixture containing DPPH (0.2 μmol/L, 2 ml) and SIPS (2 ml) was darkly incubated for 30 min. Optical density (OD) values at wavelength of 520 nm were measured. Scavenging ratio was calculated as the follows:

Scavenging ratio = (OD_C - OD_I) / OD_C × 100%

where C = absolute alcohol plus DPPH solution, I = SIPS solution plus DPPH solution.

Detection of DNA cleavage

The present DNA damage protection experiment was performed according to the reported methods that was modified slightly (Kumar and Chattopadhyay, 2007). Plasmid pEGFP-N1 DNA isolated with GeneJET™ PCR Purification Kit from Escherichia coli DH5α was used in this trial. A volume of 10 μl in a microfuge tube containing about 100 ng of plasmid DNA, H₂O₂ (100 mmol/L) and different final concentrations of SIPS were used. The reaction was initiated by UV irradiation for 2 min at room temperature. The reactive mixtures were subjected to electrophoresis in agarose gel and then stained with ethidium bromide followed by densitometric analysis.

Statistical analysis

Experimental data were analyzed by analysis of variance (ANOVA) using the JMP 7.0 statistical software. Results were expressed as the mean ± standard deviation (SD). Differences were separated by Duncan’s multiple range test. Significance was considered at p < 0.05 or 0.01.

RESULTS

In present study, we investigated antioxidant ability of SIPS in vitro. Firstly, the total reducing power of the marine bioactive polysaccharides was analyzed in this paper. Data presented in Figure 1 showed a marked reducing power of SIPS on Fe²⁺ in a dose-dependent manner, which indicated that SIPS has an ability of maintaining systemic reduction state.DPPH is a stable organic free radicals, the stability originates from spatial obstacles of benzene ring that block the pairing process of the odd electron in central nitrogen atom. Based on a strong absorption of DPPH odd electron at wavelength of
517 nm and the purple characteristic of DPPH in ethanol solution, in the presence of free-radical scavengers, the odd electrons are paired off and the absorption decreases gradually, the resultant decolorization rate is stoichiometric in accordance to the number of electrons taken up. Because of the relatively short detection time of the assay method, we measured the scavenging ability of SIPS on DPPH radicals. Results showed an increasingly quenching activity on DPPH radicals in a positive dose dependent response to the scheduled doses of SIPS (Figure 2).

Hydroxyl radical is the strongest oxidant among all of free-radicals and can almost react with any macromolecule, which leads to body damage and gene mutation resulting in aging and tumorogenesis. This experiment detected scavenging ability of SIPS on hydroxyl radical originated from H$_2$O$_2$ catalyzed by Fe$^{2+}$ using Fenton reaction. Since hydroxyl radical can decolorize bromopyrogallol red, hydroxyl radical content can be measured with Fenton reaction with respect to decolorization rate. The detection results are presented in Figure 3. The addition of SIPS produced a dose-dependent increment of scavenging activity on hydroxyl radicals.

This experiment is also designed to investigate the protective effects of SIPS on DNA damage induced by free radicals except for the aforementioned antioxidation. The protective roles of SIPS on the damage caused by H$_2$O$_2$ and UV together was performed on pEGFP-N1 plasmid DNA. Figure 4 shows the agarose gel electrophoretic pattern of DNA treated with different manner, in Figure 5 the results are expressed as percentage of optical density value of one DNA band with respect to total optical density value of three DNA bands in the plasmid lane. DNA prepared with kit from E. coli DH5α was observed with three bands in the agarose gel (lane 1), the fastest moving prominent band was supercoiled plasmid DNA (SC-DNA), the faster moving band was linear DNA (LIN-DNA) and the top faint band was the slowest moving open circular plasmid DNA (OC-DNA). Lanes 3 and 4 showed an unobvious changes compared with control DNA (lane 1), which indicated that UV irradiation or H$_2$O$_2$ did not obviously affect normal double helix structure of DNA. However both UV irradiation and H$_2$O$_2$ co-treatment resulted in marked decrease of SC-DNA and serious increase of the two abnormal DNA, LIN-DNA and OC-DNA (lane 2).

It is clear that normal SC-DNA was damaged by the combined action of the two physical and chemical factors, which showed hydroxyl radicals generated from H$_2$O$_2$ photolyzed by UV seriously cut single chain or double chains, especially double chains of SC-DNA. Also, the two figures showed that SIPS significantly impaired the damage on native SC-DNA induced by hydroxyl radicals generated from UV-photolyzed H$_2$O$_2$ in a dose-dependent manner. The low dose of SIPS (lane 5) hardly produced protective effects on the DNA damage, the percentage of densitometric units of SC-DNA in this lane were almost similar to that of the unprotected co-treated DNA in lane 2 but with the increase of SIPS concentrations, the percentage of SC-DNA increased markedly and the ratio of OC-DNA or LIN-DNA declined sharply.

Summarily, the results reveal that the marine bioactive
polysaccharides SIPS not only has strong scavenging activity on DPPH and hydroxyl radicals as well as total reducing power which are collectively called antioxidant ability, but also seriously protects DNA from oxidative damage induced by free-radicals originated from combined action of UV and H$_2$O$_2$. 

**Figure 2.** Scavenging ability of SIPS on DPPH radicals. The results are expressed as percentage of in absorbance at 520 nm with respect to control. Each value represents the mean ± SD of the four experiments.

**Figure 3.** Scavenging ability of SIPS on hydroxyl radicals. The results are expressed as percentage of in absorbance at 520nm with respect to control. Each value represents the mean ± SD of the four experiments.
Figure 4. Protective effects of SIPS on DNA damage. DNA was treated by different treatment manners for 2 min, lane 1 to 7 represent treatment manners as follows. 1: control, 2: UV + H$_2$O$_2$, 3: UV only, 4: H$_2$O$_2$ only, 5: SIPS (1.75 mg/ml) + UV + H$_2$O$_2$, 6: SIPS (7.0 mg/ml) + UV + H$_2$O$_2$, 7: SIPS (28.0 mg/ml) + UV + H$_2$O$_2$. OC, LIN and SC represent open circular, linear and supercoiled DNA, respectively.

Figure 5. Densitometric analysis of supercoiled, linear and open circular plasmid DNA after different treatment. The value was represented with percentage between densitometric unit of each DNA and total densitometric unit of three DNAs. Lane 1 to 7 represent treatment manners as follows. 1: control, 2: UV + H$_2$O$_2$, 3: UV only, 4: H$_2$O$_2$ only, 5: SIPS (1.75 mg/ml) + UV + H$_2$O$_2$, 6: SIPS (7.0 mg/ml) + UV + H$_2$O$_2$, 7: SIPS (28.0 mg/ml) + UV + H$_2$O$_2$. OC, LIN and SC represent open circular, linear and supercoiled DNA, respectively.

DISCUSSION

Since 1950s, radiation biologists have discovered that ionizing radiation acting on organism can lead water to produce reactive oxygen species which in turn result in damage of biomacromolecules, but regretfully considered the phenomenon as an instantaneous reaction all the time. Until 1968 superoxide dismutase and its derivatives functioning on scavenging ROS was found, it was realized that ROS is constantly being created and scavenged in organism which mediates ROS to normal level of free radical homeostasis (Fang et al., 2004). However, under the circumstances such as aging, diseases or special physiology, the ROS can cause body injury. For instance, cancer patient inevitably accepts clinical chemotherapy, although cyclophosphamide is one of the most successful and widely utilized antineoplastic drugs, a well-known severe side effect of the drug is wide
oxidative damage in patient body caused by cyclophosphamide-induced oxidative stress (Emadi et al., 2009). Recently, in model animals we found that cyclophosphamide seriously reduced activities of antioxidative enzymes and elevated contents of 3,4-methylenedioxyamphetamine (MDA) and nitrogen oxide (NO) in blood/organs (Liu et al., 2012; some data unpublished). So eliminating ROS is an important pathway to improve chemotherapeutic effects.

Squid ink has been proved to be a multifunctional marine bioactive substance, such as antioxidant (Sasaki et al., 1997; Chen et al., 2010), hemostasis (Xie et al., 1994), antioxidation (Liu et al., 2011; Lei et al., 2007), antiretroviral (Rajaganapathi et al., 2000) and antibacterial effect (Sadok et al., 2004) etc. In recent years, we discovered the alleviatory effects of squid ink on oxidative damage induced by cyclophosphamide (Wang et al., 2009, 2010) and polysaccharide in the dark ink was screened to be an active component. Our previous report (Liu et al., 2009, 2010) and polysaccharide in the dark ink was screened to be an active component. Our previous report (Liu et al., 2012) revealed that SIPS effectively suppressed cyclophosphamide-induced oxidative stress on partial internal organs and impaired chemotherapeutic damage, the antioxidation of SIPS was supported by another direct evidence in vitro in the present paper that showed SIPS with strong reducing power which effectively quenched hydroxyl and DPPH radicals.

It is documented that a cause of cyclophosphamide-induced DNA damage is put down to the two metabolites of the antineoplastic drug, nitrogen mustard and acrolein. The former binds covalently to DNA and induces DNA damage in the form of strand breaks through alklylation of DNA at the N7 position of guanine, DNA-DNA cross-links, both interstrand and intrastrand, as well as DNA-protein cross-links (Emadi et al., 2009), and the latter inactivates O2-methylguanine-DNA methyltransferase, a DNA repair protein. Another important reason of DNA mediated by cyclophosphamide is that although H2O2 and superoxide anion have potential cytotoxicity, hydroxyl radical generated from H2O2 and superoxide anion catalyzed by metal ions is the major oxidant causing oxidative damage in organism and is a cause of DNA strand scission in cellular damage (Wiseman et al., 1996; Guitteridge, 1984). To find natural material able to control oxidative DNA damage, the effects of SIPS on DNA cleavage were investigated. The results indicated that addition of SIPS suppressed scission of SC-DNA and formation of LIN-DNA and OC-DNA.

Summarily, our investigation unveils a direct evidence in vitro in the present paper that showed SIPS with strong reducing power which effectively quenched hydroxyl and DPPH radicals.

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Acknowledgements

The authors greatly thank the staff from Biochemistry Center of Guangdong Ocean University for their assistance in this trial. The work was supported by National Natural Science Foundation of China (31171667).

References


Antioxidant ability of squid ink polysaccharides as well as their protective effects on deoxyribonucleic acid DNA damage in vitro

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Squid ink polysaccharides (SIPS) were isolated from squid ink, a plentiful and multifunctional marine material, and were demonstrated to have amelioratory effects on cyclophosphamide-induced damage in internal organs of model animals by our previous reports. To further investigate the protective effects of SIPS on chemotherapeutic damage caused by cyclophosphamide, this paper evaluated the bioactivities of the marine polysaccharides with a view to their antioxidant ability and their protective effects on deoxyribonucleic acid (DNA) damage using tests such as hydroxyl radicals, reducing power assay and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals, and pEGFP-N1 plasmid DNA treated with ultra violet (UV) plus H₂O₂. Data revealed that SIPS not only quenched hydroxyl radicals and 1,1-Diphenyl-2-picrylhydrazyl radicals, but showed its strong reducing power and also suppressed oxidative scission on plasmid DNA strand caused by hydroxyl radicals which originated from H₂O₂ shown by UV.

Key words: Squid ink polysaccharides (SIPS), antioxidant ability, deoxyribonucleic acid (DNA) damage

INTRODUCTION

It is well known that reactive oxygen species (ROS) produced in cells may induce oxidative damage to various biomacromolecules in cells, such as polysaccharide, protein, lipid and deoxyribonucleic acid, which results in metabolic and functional disturbance in cells and in turn leads to various pathological changes, for example aging and cancer, two major problems for human originated from ROS induced oxidative stress and deoxyribonucleic acid (DNA) damage (Cerutti, 1994; Wiseman and Halliwell, 1996). Thus quenching ROS in cells must be an effective way to prevent ROS-mediated oxidative damage induced by cellular metabolism and exogenous agents, especially some oxidative drugs such as cyclophosphamide, a most commonly used chemotherapeutic agent for cancer, which is also used in the treatment of some connective tissue and autoimmune diseases, minimal lesion glomerulonephritis, and for the control of organ rejection after transplantation (Emadi et al., 2009). Owing to the strong negative effects of cyclophosphamide-induced ROS on normal tissues except for its positive roles on tumor tissues (Emadi et al., 2009), resultant insufficient dose of cyclophosphamide results in an unfavourable therapeutic effect on cancer. So, development and application of natural antioxidants would be helpful to impair oxidative damage induced by cyclophosphamide and in turn improve the therapeutic effect of the chemotherapeutic agent on tumor.

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\[
\text{Scavenging ratio} = \left( \frac{OD_{BT} - OD_{ST}}{OD_{BT} \times 100\%} \right)
\]

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The mixture containing DPPH (0.2 μmol/L, 2 ml) and SIPS (2 ml) was darkly incubated for 30 min. Optical density (OD) values at wavelength of 520 nm were measured. Scavenging ratio was calculated as the follows:

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The present DNA damage protection experiment was performed according to the reported methods that was modified slightly (Kumar and Chattopadhyay, 2007). Plasmid pEGFP-N1 DNA isolated with GeneJET™ PCR Purification Kit from *Escherichia coli* DH5α was used in this trial. A volume of 10 μl in a microfuge tube containing about 100 ng of plasmid DNA, H₂O₂ (100 mmol/L) and different final concentrations of SIPS were used. The reaction was initiated by UV irradiation for 2 min at room temperature. The reactive mixtures were subjected to electrophoresis in agarose gel and then stained with ethidium bromide followed by densitometric analysis.

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The authors greatly thank the staff from Biochemistry Center of Guangdong Ocean University for their assistance in this trial. The work was supported by National Natural Science Foundation of China (31171667).

REFERENCES

Toxic and some essential metals in medicinal plants used in herbal medicines: A case study in Pakistan

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The levels of some heavy metals in eight different medicinal plant samples along with soils were collected from two different locations from salt range of Punjab, Pakistan in order to evaluate those vital metals involved in human health implications. These plant species were: Dodonaea viscosa, Withania somnifera, Solanum nigrum, Calotropis gigantea, Mentha spicata, Paganum hermala, Cannabis sativa, Adhatoda vasica, especially those used in the treatment of diseases and their long term usage. The evaluated metals were cadmium (Cd), Nickel (Ni), Chromium (Cr) and lead (Pb). Atomic absorption spectrophotometry (wet digestion) was used for the analyses, and content of metals per sample was expressed as mg/kg. The analysis of variance revealed that there were significant effects of site variation on medicinal species in Ni and Cr contents while the opposite were observed in case of Pb and Cd at location I. The Ni concentrations found in medicinal plants varied significantly while Pb, Cd and Cr were evident as non significant at location II. There was positive non significant correlation of Pb and Cr among soil and plant while negative non significant for Ni and Cd at both locations. The findings suggest that the use of these plant species for the management of diseases will not cause heavy metal toxicity and may be beneficial to the users in cases of micronutrient deficiency, as these metals were found to be present in readily bioavailable form.

Key words: Toxicity, heavy metals, atomic absorption spectrophotometer, medicinal plant.

INTRODUCTION

Medicinal plants play a major role in the health care sector of developing nations for the management of diseases, and almost 80% population of the world directly or indirectly depends on medicinal plants and their pro-
ducts. Thus herbal preparations, drugs and medicines have a prominent role in the pharmaceutical markets and health care sector of the 21st century (Annan and Houghton, 2008). Heavy metals are important factors for the proper functioning of vital organs in the body. Iron is a component of hemoglobin and other compounds used in respiration. Heavy metals are widespread in soil as a result of geo-climatic conditions and environmental pollution even in wilderness areas of the world. Therefore, their assimilation and accumulation in medicinal plant species is obvious. Together with other pollutants, heavy metals are discharged into the environment through industrial activities, automobile exhaust, heavy-duty electric power generators, municipal wastages, refuse burning and pesticides used in agricultural practices (Jarup, 2003).

The extract of some medicinal plants like fresh juice of onion is able to reduce both acute and chronic pain as well as irritation, with a more burly effect towards inflammation (Nasri et al., 2012). Human beings, animals and plants take up these metals from all possible environments that is, through soil, air and water. Heavy metals have the tendency to accumulate in various vital plants and as well as in human organs. Since plants and animals are essential sources of micronutrients for man, either through dietary sources and various herbal preparations, so it becomes necessary to monitor the levels and fluctuations in biological mediums that are explored by man for both dietary and medicinal purposes, because deficiencies or excesses of micronutrients can be the possible threats for disease generation. Although a lot of phytochemical and bioactivity related studies have been carried out on a number of medicinal plants, however not much has been reported on different aspects of heavy metal contents of these plants (Bayor et al., 2009).

Peganum harmala L. is recognized as Syrian rue, Wild rue and Harmal. P. harmala extracts are considered vital for drug development because they are stated to have frequent pharmacological activities in the Middle East, particularly in Iran and Egypt. For a long time, P. harmala has been used in conventional medicines for the liberation of pain and as an antiseptic cause (Asgarpanah and Ramezanloo, 2012). Higher amount of different heavy metals in various plants and ultimately in animals can cause a variety of complications in animals and human beings (Parmeggiani, 1983).

The n-hexane fraction of root was disastrous to fabricate any insecticidal effect, while the chloroform fraction was useful against Callosbruchus analis and Rhyzopertha dominica with 20% mortality. The crude ethanolic extract and its ethyl acetate fraction illustrate 40% mortality C. analis and R. dominica (Alam et al., 2012). Various types of heavy metals have also been reported in plants to decline because their accumulation trends are positively correlated with loss of flora. The heavy metals in higher amount cause toxicity which may go undetected without systematic scientific studies and procedure of their monitoring.

Although the level of heavy metal has been evaluated in plants, it is easily taken up by public due to the use of plants in different local herbal medicine. These medicinal plants have been widely employed to treat different problems in human beings and other organism (Ravindran et al., 2005). Trace element ingestion, even in very small quantity, can affect not only the physiological function and health of living organisms but also demographic, distribution and reproduction, as well as survival of organism. Toxic elements like Pb and Cd are widely distributed and mobilized in environment, and human exposure to these non-essential elements has been consequently elevated (Lansdown and Yule, 1986).

At higher level of exposure of human beings, there is damage to almost all organs and organ system, most notably central nervous system, kidneys and circulatory system, leading to death at excessive levels. At very low level, biochemical, psychological and neuron, which are the heavy oral-processes, are influenced mostly (Goldstein, 1992). Pb persists to be a significant public health problem in various countries in Asia, not only between human but also among a variety of species of other organism (Hsu et al., 2006). It is necessary to know about the level of diverse heavy metal in medicinal plants used in herbal medicine. Some of the heavy metals may be lethal and these are frequently present in environmental matrices. They are establishing in soil, water and plant and readily mobilize by human activities as waste material (Larison et al., 2000).

The present study was assumed to estimate some heavy metals in generally used medicinal plants as raw drugs and other herbal medicine. Therefore, heavy metals cause a potential threat to different organism including human health (Hsu et al., 2006). Heavy metal concentrations are eminent in all the environment that is, soil, water and air in the last decades (Nriagu and Pacyna, 1988). The toxicity of heavy metal to aquatic organisms has been the focus of interest to scientist for numerous years. Important toxic metal includes Cd, Hg, Pb, Cu, Cr, Ni, Mn, Ca, Au, Ag, Li, Ce, Ga and Fe. The heavy metals find their means to water bodies through wastewater from diverse industries (Meena et al., 2008).

In view of the fact that micronutrients can be good, toxic or lethal depending on the quantity, the study also appraise the health implications of the heavy metals, based on the suggested daily intake of medicinal plant decoctions (Anonymous, 2007). Keeping in view the extensive use of medicinal plants in herbal medicine and destructive effects of heavy metals on public health, this study therefore required to set up the presence, quantity and occurrence of six heavy metals (Ni, Cr, Cd, and Pb).

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in eight medicinal plants frequently used for the treatment and prevention and execution of diseases in various regions of the world.

MATERIALS AND METHODS

Area of study

The present investigation was conducted in the sub-urban area of Kalar Kahar, Chakwal, Pakistan. Kalar Kahar is a union council and subdivision of Chakwal District in Punjab, Pakistan. It is a tourist destination located 25 km southwest of Chakwal along the motorway. It is notable for its natural gardens, peacocks and a saltwater lake. Geographically, the valley is located between coordinates 32°26' 11" to 32°41' 18" North and 71°50' 33" to 72°30' 07" East. The present experiment was carried out in an area of 200 acre. Different types of plants are planted in this land like Dodonaea viscosa, Withania somnifera, Solanum nigrum, Calotropis gigantea, Mentha spicata, Paganum hermala, Cannabis sativa and Adhatoda vasica.

Plants

A total of 8 different medicinal plant species used in the study were collected from their natural habitat and authenticated at the Department of Botany, University of Sargodha, where voucher specimen of each plant species is deposited. The leaves were air-dried for four days and later pulverized.

Sample collection and processing

Five composite samples of 8 different plants were obtained from the vicinity of Kalar Kahar, Punjab Pakistan in September, 2012 (Table 1). During present investigation, two locations were selected for the collection of soil and plant samples from 20 km radius of Kalar Kahar Lake to evaluate variation in metals from different locations associated with health implications. The samples were stored in plastic food grade containers kept at room temperature until analysis. Samples were dried at 70°C for 48 h in a hot air oven and ground prior to chemical analysis. The soil samples were collected following the procedures described by Sanchez (1976). These collected samples were air dried, stored in labeled sealed brown bags and placed in oven for 48 h at a temperature of 72°C. The plant samples were also taken from the same locations from where soil samples were collected by sterilized equipments. The collected medicinal plant samples include D. viscosa, W. somnifera, S. nigrum, C. gigantea, M. spicata, P. hermala, C. sativa, and A. vasica (Table 1). Twenty four samples of these plants were taken during sampling from each location (3 replicates of each plant). The selected samples were washed with distilled water and diluted HCl to remove dust particles and other contaminants.

Sample preparation for analysis

Heavy metal analysis was done according to Association of Analytical Communities (AOAC) (1995) for non-volatile heavy metals. For this, 1 g powder of each sample was digested in HNO₃ and HClO₄ (9:1) using the wet digestion method by heating slowly on hotplate in fume hood chamber until clear solution was obtained. The final volume of solution was made up to 25 ml with deionized water. All necessary precautions were adopted to avoid possible contamination of sample. The heavy metals under study were Lead (Pb), Nickel (Ni), Cadmium (Cd) and Chromium (Cr). Analysis was done using atomic absorption spectrophotometer AAS 6300 (Shimadzu Japan). Standard reference material of all metals (E. Merck) was used for calibration and quality assurance.

Statistical analysis

Data for different attributes were subjected to a statistical analysis using the statistical package for social sciences (SPSS) software for correlation and one-way analysis of variance worked out. Statistical significance between the mean was tested at 0.05, 0.01 and 0.001 level of probability as suggested by Steel and Torrie (1980). The bio concentration factor from soil to forage was calculated using Microsoft excel by following the formula stated by Cui et al. (2004).

Bio concentration factor = Mean metal concentration of plant / Mean metal concentration of soil

RESULTS

Soil metals

The mean square values of Pb, Ni, Cd and Cr in selected soil samples are presented in Table 2. Analysis of variance showed significant (P < 0.001) effects of site on soil Pb and Cd while its reverse was true in case of Ni and Cr at location I. The Pb and Ni concentrations found in soil varied significantly (P < 0.001) while Cd and Cr non significantly in soil samples at location II. The mean range concentrations (mg/kg) in soil were: Pb (6.80 to 7.83), Ni (0.701 to 0.78), Cd (3.45 to 4.63) and Cr (0.028 to 0.041) at location I while Pb (6.25 to 7.86), Ni (0.59 to

Table 1. The samples with symbols.

<table>
<thead>
<tr>
<th>Symbols</th>
<th>Plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1</td>
<td>Dodonaea viscosa</td>
</tr>
<tr>
<td>PS2</td>
<td>Withania somnifera</td>
</tr>
<tr>
<td>PS3</td>
<td>Solanum nigrum,</td>
</tr>
<tr>
<td>PS4</td>
<td>Calotropis gigantea</td>
</tr>
<tr>
<td>PS5</td>
<td>Mentha spicata</td>
</tr>
<tr>
<td>PS6</td>
<td>Paganum hermala</td>
</tr>
<tr>
<td>PS7</td>
<td>Cannabis sativa</td>
</tr>
<tr>
<td>PS8</td>
<td>Adhatoda vasica</td>
</tr>
</tbody>
</table>

Table 2. The mean square values of Pb, Ni, Cd and Cr in selected soil samples.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Soil</th>
<th>Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Location I</td>
<td>Location II</td>
</tr>
<tr>
<td>Pb</td>
<td>0.312*</td>
<td>0.843**</td>
</tr>
<tr>
<td>Ni</td>
<td>0.003**</td>
<td>0.005**</td>
</tr>
<tr>
<td>Cd</td>
<td>0.499*</td>
<td>0.218**</td>
</tr>
<tr>
<td>Cr</td>
<td>0.011**</td>
<td>0.021**</td>
</tr>
</tbody>
</table>
0.70), Cd (3.68 to 4.46) and Cr (0.030 to 0.040) were at location II (Table 3).

**Medicinal plants metals**

Analysis of variance of data for heavy metals in plants showed significant (P < 0.001) effects of site on plants Ni and Cr while its opposite results were observed in case of Pb and Cd at location I. The Ni concentrations found in medicinal plants varied significantly (P < 0.001) while Pb, Cd and Cr non significantly at location II (Table 2). The mean concentration ranges (mg/kg) in soil: Pb (5.78 to 6.27), Ni (3.25 to 3.64), Cd (0.025 to 0.040) and Cr (3.40 to 4.65) in medicinal plants at location I while Pb (6.28 to 6.69), Ni (3.20 to 3.62), Cd (0.066 to 0.086) and Cr (3.87 to 4.27) were at location II (Table 4).

**DISCUSSION**

The soil Pb levels ranged from 5 to 25 mg/kg as reported by Hayashi et al. (1985). These concentrations of soil Pb were lower them those earlier researches reported by Oluokun et al. (2007) in Nigeria, but higher than those found by Aksoy et al. (1999) in Turkey while studying on bio monitoring of heavy metal pollution in that region. The lead level in location soil also depend on intrinsic nature of soil and extrinsic factors through anthropogenic activities, leading to increased level of Pb far above the background level (Osweiler, 1996). The values found during the present study were at both ends higher than the critical values of 0.85 mg/kg as reported by Adriano (1986).

The present findings were much lower than values established by Govil (2001). So, there is no danger of its toxicity for medicinal plants growing therein. The recommended values of Cd in soil are 3 to 8 mg/kg (Ross, 1994). In the present study, the values of soil cadmium were found to be higher than those reported previously by Miller (1983), and almost similar values of soil cadmium have been reported by Pierce et al. (1982). Although the level of soil Cd in current investigation were lower than the toxic level suggested for plants, but the toxicosis by this element can be anticipated in this studied area. The soil chromium values were higher than the critical level of 0.02 mg/kg as reported by Anderson et al. (1973) but lower than those reported by Bergmann (1992) as 2 to 50 mg/kg. Soil Cr content reported during present study was higher than those reported in some earlier studies (Obiajunwa et al., 2002), and there is no danger of toxicity for medicinal plants in this area by this element in soil.

Mean concentration of Pb was greater than the critical level of 0.05 mg/kg reported by Tokaliogla et al. (2000). According to World Health Organization (WHO) (1998), permissible limit for Pb contents in herbal medicine is 10 ppm. The permissible limit set by Food and Agriculture Organization (FAO)/World Health Organization (WHO) (1984) in plants is 0.43 ppm. The lowest level of Pb which can cause yield reduction is 5 to 30 ppm, while the maximum acceptable concentration for food stuff is around

### Table 3. The order of accumulation of heavy metals by medicinal plants from soil.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Order of accumulation in medicinal plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb</td>
<td>PS2 &gt; PS3 &gt; PS1 &gt; PS4 &gt; PS5 &gt; PS2 &gt; PS1 &gt; PS3 &gt; PS4 &gt; PS5</td>
</tr>
<tr>
<td>Ni</td>
<td>PS2 &gt; PS1 &gt; PS4 &gt; PS3 &gt; PS2 &gt; PS3 &gt; PS4 &gt; PS1</td>
</tr>
<tr>
<td>Cd</td>
<td>PS3 &gt; PS2 &gt; PS1 &gt; PS4 &gt; PS3 &gt; PS2 &gt; PS1</td>
</tr>
<tr>
<td>Cr</td>
<td>PS1 &gt; PS2 &gt; PS4 &gt; PS3 &gt; PS2 &gt; PS1</td>
</tr>
</tbody>
</table>

### Table 4. Mean metal concentrations (mg/kg) in soil.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Site I</th>
<th>Site II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb</td>
<td>Ni</td>
<td>Cd</td>
</tr>
<tr>
<td>S1</td>
<td>7.200</td>
<td>0.730</td>
</tr>
<tr>
<td>S2</td>
<td>7.383</td>
<td>0.710</td>
</tr>
<tr>
<td>S3</td>
<td>7.466</td>
<td>0.706</td>
</tr>
<tr>
<td>S4</td>
<td>6.800</td>
<td>0.715</td>
</tr>
<tr>
<td>S5</td>
<td>7.050</td>
<td>0.766</td>
</tr>
<tr>
<td>S6</td>
<td>7.600</td>
<td>0.701</td>
</tr>
<tr>
<td>S7</td>
<td>7.833</td>
<td>0.711</td>
</tr>
<tr>
<td>S8</td>
<td>7.400</td>
<td>0.786</td>
</tr>
</tbody>
</table>
1 ppm (Neil, 1993). Pb concentration of 80 mg/kg in plants caused deleterious effects in human. The mean Pb value in plant samples were lower than those values described earlier by Oluokun et al. (2007).

The Ni concentration in the present study was below the limit described by Abassi et al. (2007). The permissible limit set by FAO/WHO (1984) in plants was 1.63 ppm. Ni is required in minute quantity for body as it is mostly present in the pancreas and hence plays an important role in the production of insulin. Its deficiency results in the disorder of liver (Kabata-Pendias and Pendias, 1992). Environmental Protection Agency (EPA) has recommended that the daily intake of Ni should be less than 1 mg beyond which it is toxic (McGrath and Smith, 1990). However, for medicinal plants, the WHO (2005) limits have not yet been established for Ni. Ni toxicity in human is not a very common occurrence because its absorption by the body is very low (Onianwa et al., 2000). Nickel exerts a potent toxic effect on peripheral tissues and on the reproductive system (Vukadinovre and Bertie, 1988). It also causes dose-related decreases in bone marrow cellularity and in granulocytomacrophage and pluripotent stem cell proliferative responses. Pb and Nickel were present in lower amount in the plants studied as compared to the other heavy metals in medicinal plants. There, levels were lower than the permissible limits which is 10 mg/kg for Pb (WHO, 1993), and 5 to 15 mg/kg for Ni per day (Barceloux, 1999).

Cd level was much higher than the critical level suggested by National Research Council (NRC) (1980). Cadmium accumulates in human body and damages mainly the kidneys and liver. The lowest level of Cd which can cause yield reduction is 5 to 30 ppm, while the maximum acceptable concentration for food stuff is around 1 ppm (Neil, 1993). The permissible limit set by FAO/WHO (1984) in edible plants was 0.21 ppm. The obtained cadmium levels in samples in this study were below the provisional tolerable weekly intake (PTWI) presented by WHO (60 μg/day/60 Kg) (Herrman and Walker, 1999).

Cadmium is a non essential trace element with uncertain direct functions in both plants and humans (Cui et al., 2004). It is however reported that the lowest level of cadmium which can cause yield reduction in plants is 5 to 30 μg/g, and the maximum acceptable concentration for foodstuff is about 1 μg/g (Hayashi et al., 1985). The results of this study indicated that about 80% of the plant species had cadmium content above 30 μg/g which is essential for improved yield. Cd in all the medicinal plants was lower than the toxic limits which is generally accepted and the normal Cd levels in medicinal plants are between 0.2 to 0.8 mg/kg, and toxic levels of Cd were describe as 1 to 30 mg/kg (Kabata-Pendias, 1992). It has also been reported that provisional tolerable weekly intake are of Cd is 7 mg/kg/body weight/week (WHO, 1993). The values found in our study were much lower than the above described values. Higher level of Cr in plants could cause toxicities in human. The permissible limit set by FAO/WHO (1984) in edible plants was 0.02 ppm. In present study, Cr level was low from the toxic level observed in salt range of Pakistan (Ahmad et al., 2009). The toxic effects of Cr intake is skin rash, nose irritations, bleeds, upset stomach, kidney and liver damage, nasal itch and lungs cancer (McGrath and Smith, 1990). The daily intake of Cr (50 to 200 μg) has been recommended for adults by US National Academy of Sciences (Watson, 1993). Cr was found below their recommended daily allowances that are 18 mg/kg/day given by National Research Council (Anonymous) (1989). The contents of Cr range from 3.40 to 4.65 mg/kg in different plant sample.

Weak levels were similar to the results previously reported by various researchers (Koc and Sari, 2009; Maiga et al., 2005; Sheded et al., 2006). These plant cadmium were lower the Food and Drug Administration (FDA) recommended daily intake of this element for dietary sources which is 0.12 mg/g (Haider et al., 2004). This study was an attempt to enrich knowledge about the mineral contents of some medicinal plants and soil, and it may help in formulation of chemically pure medication. In this study, all of the determined values are below the WHO’s permissible levels, except Cr, and may not constitute a health hazard for consumers, so it can be recommended that medicinal plants growing in this specific area of Pakistan are suitable for phytotherapeutical uses.

Bioconcentration factor

The bioconcentration factor for Pb, Ni, Cd and Cr from soil to medicinal plants ranged from 0.8471 to 0.932, 4.367 to 5.189, 0.0159 to 0.021 and 102.79 to 149.57, respectively (Table 5). The lowest value showed that the plant may be resistant to metals while the highest value indicated highly sensitive nature of plant ( Alam et al., 2003). The low concentrations may be due to the presence of some other metals in the soil and this can suppress the uptake of metals by studied medicinal plants (Lokeshwari and Chandrappa, 2006). The bio concentration of metal depends upon the bioavailability of metals. The current investigation also supports the results that accumulation of Pb and Ni is comparatively less than that of Cd and Cr in medicinal plants (Onaniya et al., 1998).

Correlation

There was positive non significant correlation of Pb ($r = 0.081$) and Cr ($r = 0.146$) among soil and plant with negative non significant for Ni ($r = -0.236$) and Cd ($r = -0.372$) at both locations (Sinha et al., 2006). These results
showed the mineral imbalance of Pb and Cr that had strong relation among soil and medicinal plants (Fytianos et al., 2001). Considering the Ni and Cd, there was weak association between soil and medicinal plants at both locations. The overall results indicated clearly that heavy metals are present in Pakistan medicinal plants and that the contents of these metals except iron were within acceptable and safe limits. Therefore, herbal formulations of these plant species can also be beneficial sources of appropriate and essential trace elements, though care must be taken to avoid iron toxicity, especially in higher doses.

Conclusion

The present study gives a new perspective about the presence of some heavy metals (Pb, Ni, Cd and Cr) in the medicinal plants and their corresponding soil. The lead, nickel and cadmium concentrations during the present study were below the WHO permissible levels and may not constitute a health hazard for consumers in this area. The current investigation showed that the Cr concentration is above the standard level and it can be toxic for humans. Effluents can be a source of this increase. Most of the plant species contained safe levels of the heavy metals analyzed and hence may have no adverse effects normally associated with heavy metal toxicity on people who patronize these products for their health needs.

REFERENCES


<table>
<thead>
<tr>
<th>Medicinal plants</th>
<th>Site I</th>
<th>Site II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pb</td>
<td>Ni</td>
</tr>
<tr>
<td>Dodonaea viscosa</td>
<td>6.241</td>
<td>3.641</td>
</tr>
<tr>
<td>Withania somnifera</td>
<td>5.970</td>
<td>3.485</td>
</tr>
<tr>
<td>Solanum nigrum</td>
<td>5.788</td>
<td>3.378</td>
</tr>
<tr>
<td>Calotropis gigantea</td>
<td>5.970</td>
<td>3.196</td>
</tr>
<tr>
<td>Mentha spicata</td>
<td>5.761</td>
<td>3.258</td>
</tr>
<tr>
<td>Peganum harmala</td>
<td>6.270</td>
<td>3.290</td>
</tr>
<tr>
<td>Cannabis sativa</td>
<td>6.093</td>
<td>3.263</td>
</tr>
<tr>
<td>Adhatoda vasica</td>
<td>5.780</td>
<td>3.196</td>
</tr>
</tbody>
</table>


Assessment of copper intensity in selected tissues of two different classes of ruminants in Punjab, Pakistan

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INTRODUCTION

It is a pre-requisite for productivity and the health of ruminants to provide them an appropriate quantity of minerals (Khan et al., 2006). The cattle get minerals by grazing forage plants but imbalanced mineral intake adversely affect their rate of reproduction and productivity (Khan et al., 2007). It is observed that copper is an essential component of many metalloproteins or metalloenzymes that is, superoxide dismutase, lysyl

Key words: Tissue, serum, buffaloes, cattle, plant, soil, animal, mineral.
oxidase, ceruloplasmin, metallothionein and cytochrome c oxidase, so it is responsible for various oxidative processes (Minute and Carfagnini, 2002). The toxicity of copper causes oxidative damage, renal failure, distortion of erythrocytes and eventually sudden death due to the rapid emission of copper from different sites of animal liver (Underwoodand Suttle, 1999). Copper is a cofactor of many cuproenzymes, and recent research has identified several proteins involved in its metabolism (Harris, 2000). Ruminants are particularly susceptible to copper toxicity, whereas monogastric species are usually quite tolerant. Copper toxicity in cattle is associated with excessive intake of copper in the diet or contamination of pastures by soils, slurry, mining or industrial emissions and waste (Lopez-Alonso et al., 2002; Miranda et al., 2005). It was also observed that the concentrations of Cu in the liver were correlated with the Cu content in the soil (Lopez-Alonso et al., 2003; Miranda et al., 2006).

It was observed that there were no significant difference between male and female buffaloes and cows, but male animals have lower copper concentration in blood-serum than that of female animals. In polluted areas, copper levels increased with age, in the liver of buffaloes and cows. There is a competition between binding sites of metallothionein, which become a cause of antagonistic interaction of zinc (Zn), copper (Cu) and cadmium (Cd). Cu play an important role in increasing the rate of heart beat, bone production and connective tissue development etc. Heart diseases, anemia, and change of hair color, diarrhea, infertility and also low antibodies production, was mostly observed in ruminants, which have copper deficiency. The excess of copper accumulation shows toxicities, which may cause many severe diseases, such as collapse, abdominal pain, nausea, vomiting and paralysis. Hypercupremic occur due to increasing copper concentration, as a result of which decreases the rate of reproduction and growth and also cause muscular dystrophy. The adverse effect was experienced in buffaloes and cows due to calving, which is also called reproductive disorders (El-Wishy, 2007).

Copper plays a number of important biological roles in animals through several Cu-dependent enzymes (Xin et al., 1991). Copper deficiency in ruminants occurs either as a primary or as a secondary deficiency. Most of the copper deficiencies in livestock which occur naturally are conditioned by the presence of dietary factors that interfere with the absorption or utilization of copper by the animal (Underwood and Suttle, 1999). These dietary factors, such as iron, molybdenum or sulfur, interfere with the absorption and metabolism of copper (Suttle, 1991). In the ruminants, molybdenum combines with reduced sulfur to form tetrathiomolybdate that binds copper to prevent its absorption, while other thiomolybdates and molybdates are absorbed into blood and bind endogenous copper to render it unavailable for metabolic purposes (Mason, 1982).

Copper deficiency has been reported in grazing livestock in some parts of Iran (Nouri, 1998; Nouri et al., 2005). In the west and east Azerbaijan and Kurdistan provinces, high molybdenum levels were responsible for copper deficiency among ruminants. Swayback in lambs and kids in the Khuzestan province of Iran has been observed as frequently (Nouri et al., 2005) reported enzootic ataxia in lambs in this region. There is no information with regard to copper deficiency in goats in Khuzestan province; therefore the aim of this study was to determine serum and liver copper concentrations of goats in some parts of the area and to evaluate the effects of antagonistic minerals for the determination of primary or secondary copper deficiency. Trace element deficiencies are more common in combination than single element deficiency (Kankofer, 2000).

Low level of copper, iron, and selenium (Grys and Kubinski, 1979; Sobiech and Kuleta, 2002; Faixova et al., 2007; Bickhardt et al., 1999) and low levels of selenium and zinc (Gupta et al., 2004; Han and Kim, 2005) have been reported in cases of retention of fetal membranes as compared to control values in cows. Significant low level of zinc has also been reported in the plasma of cows with calving difficulty (Dufty et al., 1977) and in buffaloes with uterine prolepses (Kelkar et al., 1989; Bhatti et al., 2006). Trace elements of metals such as manganese (Mn), copper (Cu), iron (Fe), selenium (Se) and zinc (Zn) are essential in animal nutrition and are needed in very small amounts for essential metabolic reactions in the body. Their deficiencies are often associated with alterations in many metabolic processes and cause various kinds of diseases.

Deficiency of these trace elements causes severe economic loss due to increased susceptibility to oxidative stress, growth retardation in young animals, anemia (Bureau et al., 2003), decrease in feed efficiency and fertility (Grenier et al., 2003), enhance the virulence of the infectious agent (Failla, 2008) and decrease immune system function (Rink and Ibs, 2003). On the other hand, normal thyroid status is dependent on the presence of many trace elements such as Se, Fe, Cu and Zn, for both the synthesis and metabolism of thyroid hormones. Although the role of some of these elements such as iron, zinc and copper in the thyroid are less well defined, sub or super optimal dietary intakes of these elements can adversely affect thyroid hormone metabolism (Nazifi et al., 2009).

The purpose of present study was to determine the mineral status (copper) of animals, we can determine the mineral requirements for ruminants and economic benefit for mineral supplementation, so that we can easily adopt various strategies to overcome the problems of mineral imbalances in soil and plants consumed by animals.

MATERIALS AND METHODS

This study was designed to determine the level of micro-mineral copper in liver, kidney, ribs, cerebrum, blood-serum, lungs, bones...
and hair of cows and buffaloes at district Jhang, Toba and controlled farm (Ever Green livestock farm Bukhar road District Jhang), and also to determined its level in the fodder and soil of the above given locations. Samples from free grazing cattle were obtained from abattoirs situated in widely spread localities in Punjab, Pakistan. The animals were not selected according to sex or age but on the acknowledged assumption that they were aged from 2 to 5 years, and taking of the parts that reach the final consumers. The samples were packed in polyethylene bags and conveyed to the laboratory. Upon reception, gross fat was removed and stored at -10°C in sealed plastic container until required. Samples of various organs were collected from 60 animals, consisting of 12 cows and 12 buffaloes (district Jhang), 12 cows and 12 buffaloes (district Toba) and 6 cows and 6 buffaloes from experimental and controlled farms, respectively.

Sample collection

The work was conducted in different regions of two districts of Punjab, Jhang and Toba. Soil, fodder and animal samples were collected from fields of Jhang, Toba and controlled farm. The soil samples were obtained from those fields where fodders were cultivated. Soil samples were collected from each selected field and controlled farm from different surfaces up to 15 to 20 cm depth at three different points by using a stainless steel sampling augur, the samples were air dried, ground and mixed. 10 g of air dried soil was added in 125 ml conical flask along with 40 ml mehlich-extracting solution (0.05 N HCl + 0.025 N HClO4), then shaken for 15 min in a reciprocal shaker. The extract was then filtered through Whatmann filter paper No. 2 and stored in labeled clean plastic bottles for further laboratory analysis.

The samples of various fodders, which were being fed as such to the buffaloes, were collected from both districts from the owners of animals. A total of 300 fodder samples were collected. Fodder frequently grazed by cows and buffaloes are turnips, sugar cane tops, berseen, maize, local weeds, barley, paddy straw, wheat straw, tree leaves, grass and sorghum etc. Forage samples were collected at 15 cm from the ground, by hand plucked method, which is grazing behavior of animals. The samples were washed with 1% HCl followed by 3 to 4 washing. With double distilled water, and air dried and then oven dried at 65°C, these samples were ground in a powder form, then 1 g of the dried forage sample was added in a 50 ml conical flask, added 5 ml concentrated HNO3 along with 5 ml HClO4, then these samples were digested on hot plate at high temperature till volume reduced to 1 to 2 ml. The material was allowed to cool down, the contents were filtered and diluted unto 50 ml with double distilled water and stored in clean, air tight labeled bottle for copper mineral analysis.

A total of 480 animal tissue samples (blood-serum, cerebrum, blood-serum, cerebrum, blood-serum and hair) which are considered excellent mineral status indicating organs, were collected in triplicate from each of 30 cows and buffaloes (both male and female, aging between 2 to 13 years) after knowing their origin, from different fields and control farm at two districts, viz, Jhang and Toba Tek Singh, Punjab, Pakistan. Blood samples were collected from the jugular vein of animals into 20 ml sterilized plastic tubes heparinized with 6 drops of heparin as anticoagulant. The heparinized blood samples were stored at -20°C and were allowed to stand for approximately 3 h in a slanting position at room temperature. These samples were centrifuged for 10 min at 3000 rpm by Micro centrifuge, to get blood-serum. After labeling, the serum sample was stored in refrigerator in contaminant free tubes. After collection of blood samples, the above-mentioned organ samples (60 g) of the same animal were also collected. These organ samples were dried in an oven after placing them in labeled paper envelope for several hours till complete dryness. Dried sample was ground to a fine powder so that they can easily be digested. Atomic absorption spectrophotometer requires clear, organic matter free and transparent solution. For this purpose, wet digestion was carried out.

Digestion of samples

For wet digestion, 2 ml of blood sample and 2 g of each of various organs samples were taken in a conical flask and 20 ml of acid mixture in the ratio of 1:5:2 (H2SO4: HNO3: HClO4) added. The solution was heated first at low temperature and then at high temperature on a hot plate until the whitish fumes come out (that is, all the organic matter dissolved) and 2 to 3 ml of clear, transparent solution left behind in the flask. Then after cooling, de-ionized water was added to make 60 ml of solution and this solution filtered through whatmann filter paper #42 to get transparent filtrate, which was preserved in labeled sample bottles. These digested and diluted samples were used for the estimation of copper element (Kamada et al., 2000). Standard solution of Cu micro mineral was prepared by using available standard salt (copper sulphate) of copper to get standard curve of absorbance value of this mineral. Finally, the copper concentration in each of the sample was calculated. For blood-serum and organs of animal copper, concentrations were calculated in mg/L and mg/kg, respectively.

Analytical procedure

The above samples of soil, forage, animal organs and blood-serum were diluted as required and analyzed for copper metal by using atomic absorption spectrophotometer, according to the method reported by Anwar et al. (2004).

Statistical processing

The values obtained from the studied indicators were processed by using the general linear model STATISTICA (StatSoft Inc., 7.1) procedure. The differences among means were ranked using Duncan's new multiple range test (Duncan, 1955).

RESULTS

Mineral values in soil, forages, animal organs and their blood-samples obtained in present study were compared with those of controlled farm and also with reference values already present in literature to determine the various categories of levels of mineral imbalances. The results pertaining to concentration of copper in various tissues of cows and buffaloes (liver, kidneys, ribs, cerebrum, blood- serum lungs, bones and hair) are shown and their levels are presented as mean ± SE values, and augmented with bar charts and tables. Analysis of variance for copper concentration in liver, cerebrum, blood-serum, lungs, bones and hair showed significant results (p < 0.05) of the district and age of the male cows, but the reverse was true for kidney and ribs, in which copper concentration was non significant (p > 0.05) (Table 1).

The mean ± SE copper concentrations (21.28 ± 1.23, 28.06 ± 0.62, 27.03 ± 2.63 mg/kg, 21.43 ± 0.94 mg/l, 22 ± 1.33, 19.38 ± 1.52 and 11.03 ± 0.38 mg/kg) and its
concentrations (27.13 ± 1.30, 29 ± 3.85, 25.21 ± 1.97 mg/kg, 22.51 ± 3.02 mg/l, 29.77 ± 4.05, 16.16 ± 2.80, and 16.2 ± 1.53 mg/kg) in liver, kidney, ribs, blood-serum, lungs, bones and hair of male cows of both districts Jhang and Toba, were higher as compared to the corresponding concentrations (5.86 ± 0.37, 6.03 ± 0.48, 9.29 ± 0.48, 5.8 ± 0.86 mg/l, 4.71 ± 0.49, 7.35 ± 0.57 and 6.79 ± 0.53 mg/kg) of control groups, respectively (Figure 1a). Analysis of variance of data for copper concentration in liver, kidney, ribs, cerebrum, blood-serum, lungs, bones and hair showed significant result (p < 0.05) of the district and age of the female cows (Table 2). The mean ± SE copper concentrations (18.69 ± 1.21, 24.75 ± 1.38, 21.44 ± 1.84 mg/kg, 20.54 ± 1.06 mg/l, 21.73 ± 1.32, 20.72 ± 2.67 and 11.3 ± 0.53 mg/kg) and its concentrations (29.39 ± 2.09, 26.71 ± 1.89, 25.58 ± 2.93 mg/kg, 28.8 ± 2.56 mg/l, 21.53 ± 2.03, 15.07 ± 2.58 and 14.53 ± 2.06 mg/kg) in liver, kidney, ribs, blood-serum, lungs, bones and hair of female cows of both districts Jhang and Toba were higher as compared to the corresponding concentrations (5.99 ± 0.86, 7.5 ± 0.49, 7.9 ± 0.64 mg/kg, 4.72 ± 0.54 mg/l, 3.28 ± 0.55, 6.32 ± 0.56 and 7.00 ± 1.06 mg/kg) of control groups, respectively and lower for cerebrum (Figure 1b).

Analysis of variance of data for copper concentration in liver, kidney, ribs, cerebrum, blood-serum and lungs, showed significant result (p < 0.05) of the district and age of the male buffaloes, but reverse was true for bones and hair, in which copper concentrations were non significant (p > 0.05) (Table 3). The mean ± SE copper concentrations (10.62 ± 0.84, 22.06 ± 1.17, 24.34 ± 0.70 and 11.42 ± 0.59 mg/kg) and its concentrations (6.83 ± 1.39, 20.86 ± 0.99, 26.64 ± 1.19 and 11.34 ± 0.90 mg/kg) in liver, kidney, ribs, and bones of male buffaloes of both districts of Jhang and Toba were higher as compared to the corresponding concentrations (23.99 ± 2.64, 27.3 ± 1.20, 29.54 ± 2.05 and 19.46 ± 3.35 mg/kg) of control groups, respectively but reverse was true for liver, kidney, ribs and bones (Figure 2a).

Analysis of variance of data for copper concentration in liver, kidney, ribs, cerebrum, blood-serum, lungs, bones and hair showed significant result (p < 0.05) of the district and age of the female buffaloes (Table 4). The mean ± SE copper concentrations (10.02 ± 0.97 mg/kg, 20.19 ± 1.17 mg/l, 24.01 ± 0.76, 13.89 ± 0.82 and 12.44 ± 0.85 mg/kg) in cerebrum, blood-serum, lungs, bones and hair of female buffaloes of district Jhang were lower as compared to the corresponding concentrations (23.36 ± 2.42 mg/kg, 25.33 ± 1.28 mg/l, 27.03 ± 1.40, 15.26 ± 0.71 and 16.02 ± 1.64 mg/kg) of control groups, respectively and the reverse was true for liver, kidney and ribs and its concentrations (23.58 ± 1.66, 73.66 ± 1.58, 27.3 ± 1.15 and 18.84 ± 1.16 mg/kg) in kidney, ribs, lungs and bones of female buffaloes of district Toba were higher as compared to the corresponding concentrations (17.13 ± 1.01, 33.38 ± 0.50, 27.03 ± 1.40 and 15.26 ± 0.71 mg/kg) of control groups, respectively and the reverse was true for liver, cerebrum, blood-serum and hair (Figure 2b). The mean ± SE copper levels for the fodder samples from the fields of Jhang and Toba were 22.18 ± 0.45 and 22.86 ± 0.31 mg/kg, respectively, and for these samples from the control farm were 22.72 ± 0.27 mg/kg. Copper concentration in fodders was slightly lower at districts Jhang and slightly higher at Toba as compared to those of control farm, respectively (Figure 3a). The mean ± SE copper levels for the soil samples from the fields of Jhang and Toba were 2.84 ± 0.11 and 3.62 ± 0.20 mg/kg, respectively, and for the soil samples from the control farm were 1.82 ± 0.20 mg/kg. Copper concentration was higher in soil of both districts Jhang and Toba as compared to that of control farm (Figure 3b).

**DISCUSSION**

In the present study, the mean ± SE copper levels in liver, kidney, ribs, blood-serum, lungs, bones and hair of both male and female cows of both districts were higher as compared to those of control groups but slightly lower than those of earlier reported reference values, but reverse was true for the cerebrum. Erdogen et al. (2002) also studied lower copper mineral contents in blood of cattle. They concluded that copper mixtures should be continuously added to the fodders of cattle. The slight difference of copper levels in various groups of cows and buffaloes was due to the variation of digestibility of copper from their diets, fluctuation in the climatic conditions, soil chemistry or nature of forages.
Table 2. Analysis of variance of data for Cu concentration in various organs of female cows of different age groups at two districts of Punjab.

<table>
<thead>
<tr>
<th>Source of variation (SOV)</th>
<th>Degree of freedom (df)</th>
<th>Mean square (female cows)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Districts (D)</td>
<td>1</td>
<td>1030.4*</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>1.25</td>
</tr>
<tr>
<td>Ages (A)</td>
<td>5</td>
<td>92.92*</td>
</tr>
<tr>
<td>D×A</td>
<td>5</td>
<td>44.9*</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* = Significant Level at 0.05 levels; ns = non-significant levels.

Table 3. Analysis of variance of data for Cu concentration in various organs of male buffaloes of different age groups at two districts of Punjab.

<table>
<thead>
<tr>
<th>Source of variation (SOV)</th>
<th>Degree of freedom (df)</th>
<th>Mean square (male buffaloes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Districts (D)</td>
<td>1</td>
<td>6.674*</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>0.167</td>
</tr>
<tr>
<td>Ages (A)</td>
<td>5</td>
<td>57.946*</td>
</tr>
<tr>
<td>D×A</td>
<td>5</td>
<td>54.522*</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>0.765</td>
</tr>
</tbody>
</table>

* = Significant Level at 0.05 levels; ns = non-significant levels.

Figure 1. Fluctuation in levels of Cu in liver, kidney, ribs, cerebrum, blood-serum, lungs, bones and hair of (a) male cows and (b) female cows.
Table 4. Analysis of variance of data for Cu concentration in various organs of female buffaloes of different age groups at two districts of Punjab.

<table>
<thead>
<tr>
<th>Source of variation (SOV)</th>
<th>Degree of freedom (df)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Ribs</th>
<th>Cerebrum</th>
<th>Blood-serum</th>
<th>Lungs</th>
<th>Bones</th>
<th>Hair</th>
</tr>
</thead>
<tbody>
<tr>
<td>Districts (D)</td>
<td>1</td>
<td>11.696*</td>
<td>30.104*</td>
<td>1038.8*</td>
<td>620.43*</td>
<td>59.008*</td>
<td>97.904*</td>
<td>8.102*</td>
<td>4.114*</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>0.453</td>
<td>0.518</td>
<td>2.28</td>
<td>0.423</td>
<td>0.844</td>
<td>0.883</td>
<td>0.16</td>
<td>0.33</td>
</tr>
<tr>
<td>Ages (A)</td>
<td>5</td>
<td>72.586*</td>
<td>135.67*</td>
<td>506.32*</td>
<td>47.236*</td>
<td>39.555*</td>
<td>38.147*</td>
<td>37.227*</td>
<td>30*</td>
</tr>
<tr>
<td>D x A</td>
<td>5</td>
<td>72.078*</td>
<td>18.637*</td>
<td>648.42*</td>
<td>89.934*</td>
<td>79.301*</td>
<td>16.964*</td>
<td>21.94*</td>
<td>26.834*</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>0.765</td>
<td>0.417</td>
<td>232.24</td>
<td>0.554</td>
<td>0.658</td>
<td>0.673</td>
<td>0.69</td>
<td>0.372</td>
</tr>
</tbody>
</table>

* = Significant Level at 0.05 levels; ns = non-significant levels.

Figure 2. Fluctuation in levels of Cu in liver, kidney, ribs, cerebrum, blood-serum, lungs, bones, and hair of (a) male buffaloes and (b) female buffaloes.
In present study, the copper levels in liver, kidney and ribs and bones were higher but lower in blood-serum, cerebrum, lungs and hair of male buffaloes in experimental sites of both districts, and also significantly lower in cerebrum, blood-serum, lungs, bones and hair of female buffaloes of Jhang. Muehlenbein et al. (2001) also reported the lower levels of copper in blood-serum of buffaloes, but these results showed no agreement with those reported by Kankoffer (2000). The cause of copper deficiency was due to high intake of molybdenum and sulphur which showed antagonistic behavior among these minerals. In this study, the higher levels of copper in liver, kidney and ribs of male buffaloes than those of earlier reported reference values indicate more accumulation of organic materials in the soil.

The analysis of fodders of both districts and control form under study indicated that the fodders of district Toba were slightly deficient in copper minerals. Akhtar et al. (2009) also reported slightly lower copper levels in fodders of fields as compared to control farm. In blood-serum and other animal tissues of all classes of animals at given experimental sites, it showed copper deficiency except liver, kidney and ribs. Copper element deficiency was more common in combination than single element deficiency, which became a cause of retention of fetal membrane (Kankoffer, 2000). Muhlenbein et al. (2001) reported that the cows showed higher copper concentration by 24 mg/kg than that of control groups when these cows were supplied with supplementation of organic materials. Copper deficiency adversely affects the immune system by decreasing number of antibody producing cells (Chew, 2000). Copper availability of plants from soil may be affected by soil pH. Actually, its availability in plants increased by decreasing soil pH, so its availability in plants was also highly dependent on the amount and kind of organic matter in the soil. The study on cows and buffaloes had rather equal serum copper while more serum zinc, which was about 5 times higher (Nazifi et al., 2009).

**Conclusion**

The findings of this work showed that the distribution of minerals in different organs of ruminants depended upon the rate of absorption through gastro intestinal tract of
grazing animals. By knowing the mineral status of animals, we can determine the mineral requirements for ruminants and economic benefit for mineral supplementation, so that we can easily adopt various strategies to overcome the problems of mineral imbalances in soil and plants consumed by animals. It is recommended to provide additional copper in a concentrated mixture form to the animal nutrition for the correction of copper deficiency. Copper deficiency in animals can be prevented by using intramuscular injection which is a slowly absorbed form of copper. On the basis of the information obtained from soil, pasture forages, blood-serum and various organs, it is possible that low levels of zinc and copper in soil and forage could potentially limit ruminant reproduction. It is concluded that mineral supplement should continually be supplied to the grazing ruminants to improve the mineral status for maximizing the production potential of livestock at the sites of these districts of Punjab.

REFERENCES


Chew BP (2000). Micronutrients play role in stress and production in grazing ruminants to improve the mineral status for maximizing the production potential of livestock at the sites of these districts of Punjab.


Ethnobotanical survey on plants of veterinary importance around Al-Riyadh (Saudi Arabia)

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Ethnobotanical survey was conducted on ethno-veterinary medicinal plants used by people living in Al-Riyadh, Saudi Arabia. The aim of the study was to prepare an inventory of medicinal plants used for the curing of different livestock ailments. Ethno-veterinary knowledge of local people was collected through questionnaire and interviews during field trips. Fifty-two plant species were identified as being used for treatment of 10 common livestock ailments. The methods and application of uses of these plants varies based on the nature of animal disease, and their veterinary significance was briefly reviewed. The study concluded that the medicinal plant knowledge of the people is strongly influenced by Arab system of traditional medicine. Secondly, distinct local use of plants also existed which indicated that plant knowledge specific to the local Bedouins is still alive and practiced. Most of the plants mentioned in the current paper are traditionally used for the treatment of different animals’ diseases and it is essential to be mindful of their toxic potential and possible side effects.

Key words: Medicinal plants, livestock, diseases, traditional medicine, ethno-veterinary.

INTRODUCTION

The climate of Saudi Arabia can be characterized as arid (Azaiz et al., 2003; Al-Quran, 2008). Ecologically, such zones are fragile and difficult to develop and use. Except in limited areas, the vegetation of arid areas is sparse and usually highly specialized both morphologically as well as physiologically. Hence, this region has been regarded as a natural reservoir for the collection of a variety of wild medicinal and aromatic plants (Sher et al., 2010a; Muhammad et al., 2012).

Gathering and processing of medicinal and aromatic plants for family use in human and livestock treatment is centuries old practice, and have also been used virtually in all cultures (Sher and Al-yemeni, 2010b). The use of traditional medicine for maintenance of health in most of the developing countries has been widely observed as a custom. Furthermore, an increasing reliance on the use of medicinal and aromatic plants in the developed societies has been traced to the extraction and development of several drugs and chemo therapeutics from these plants, as well as from traditionally used rural herbal remedies (Jinous and Fereishteh, 2012). Moreover, in these societies, herbal medicines have become more popular in the treatment of minor ailments and also on account of the increasing costs of personal health maintenance.

Ethno-veterinary medicine is the study of indigenous knowledge systems of animal health care, the practical aspects of which are relevant to applied development work. The people manage and conserve significant amounts of biological resources useful for human, veterinary, industry and world community (Hussain and Sher, 2005; Ozcan, 2005; Al-Quran, 2008; Sima et al., 2012). However, it has been noticed that local people lack systematic approach in different scientific aspect.
such as: anthropological, social, chemical, pharma-
cological, botanical, ecological, agronomic, habitat loss of
plant species survival, their genetic depletion, documenta-
tion and others (Sushila et al., 2010; Al-Quran, 2008).
No doubt, a significant governmental support, resources,
and involvement of skilled personnel are needed. In
addition, during the last three decades, it has become a
global trend to replace allopathic drugs having different
side effect in humans and animals, by relatively safer na-
tural drugs. The turning point was reached when people
became aware of reports such as thalidomide/thalomid

The traditional practice of extracting medicinal plant
resources is mostly unsustainable. There is a lack of
resource tenure and custodianship, understanding of
sustainable use and management parameters, proper
harvesting and collection procedures and knowledge of
market requirements. These are important barriers to the
sustainable utilization of medicinal and aromatic plants
besides scientific understanding of population size, distri-
bution, availability and abundance of plant species and
their interaction with different stochastic. Keeping in view
the misuse of natural resources, there is great need to
prevent the flora and take measures to ensure
sustainable availability of different plants for human and
veterinary use (Ali et al., 2002; Saganuwan, 2010).

Therefore, the present endeavour was initiated with the
aim to prepare an inventory of medicinal plants uses for
the treatment of different livestock ailments in Arab folk
medicine.

MATERIALS AND METHODS

Study area

The study area "Al-Riyadh" (24° 38' 27" N, 46° 46' 22" E) is located
in the central of Arabian Peninsula and is home of 7 million people
(Figure 1). Al-Riyadh area has a mean maximum-minimum air
temperature varying from 44.2°C during July to 7.6°C during
January (Figure 2). The climate of the area is dry arid type and the
rainfall is unpredictable in the Al-Riyadh area; where the mean
maximum amount of rainfall are 29.2 mm during March to zero mm
as a minimum amount during the summer season. Rough and
rugged terrain and extreme climatic conditions have developed
certain fragile habitats, which are exposed to rapid degradation due
to ever increasing population pressure.

Survey procedure

The ethnobotanical survey was conducted on ethno-veterinary
medicinal plants during summer 2010 in various parts Al-Riyadh of
Saudi Arabia. A semi-structured questionnaire was devised to
document the traditional knowledge of local people regarding
medicinal plants (CRP 1992; Raziq et al., 2010). Generally, elder
persons whose practical knowledge was respected by others and
those who practice popular folk medicines for the curing of different
livestock diseases were contacted and interviewed about the
plants. Information about the local names, local uses, parts used,
and time of collection, processing and recipe preparation were

RESULTS

The current survey generally observed that the farmers
within the community of the study area possessed a
sophisticated body of ethno-veterinary knowledge about
animal diseases, treatments for animal diseases, and
animal management practices. The study revealed that
there were total 52 plants species used in ethno-
veterinary practices by the local people rearing different
livestock especially cows, goats, sheep and camels. These 52 plants species were found to be distributed
among 26 families, out of which 24 were dicotyledons
and 2 were from monocots families.

The results of the current study on the medicinal plants
of ethno veterinary importance demonstrated that such
plant species might be used as a single recipe while
some others were mixed with other plant species and
used in animal health care. Similarly, few plants species
were considered for the treatment of only one specific
disease while several other plant species were utilize for
multiple purposes. The results of present study are
depicted in Table 1 and Figures 3 and 4 which docu-
ments plant remedies addressing 10 different diseases,
their therapeutic indications and pharmacological
activities. The most common mentioned ailments were:
urinary tract infection, digestive system, fever, cold or flu
inflammations, flatulence, removal of external parasites,
carinative, deworming and appetizer agent. The main
uses coincided well with the descriptions given in Arab
traditional system of medicine. However, some additional
uses of the remedies were also observed in the study
area.

DISCUSSION

The forest, grassland and even agriculture land of the
study area support a number of plant species, of which
many have potential economic, medicinal and other
ethno-botanical values. Majority of the people living within
and around the study area rely on the plant resources for
centuries. The study showed that the local people have
rich indigenous knowledge about distribution, abundance,
harvesting, uses and marketing of medicinal and
aromatic plants. The indigenous knowledge however
differs in extent among gender, occupational and social
groups.
Figure 1. Map of the study area.

Figure 2. Climatic data of the study area.
The result of the present investigation can be applied to agriculture forestry in general and to pharmaceutical industry in particular. The inhabitants of the area get significant benefits from forest which comes mainly in the form of vast array of forest products, most of which are non timber forest products especially medicinal plants. In this context, the folklore ethno-veterinary knowledge deals with the traditional uses of plants in the indigenous system for curing of different livestock ailments. The present study area is rich in plant diversity, which is most significantly associated with the life and practices of the hill side dwellers of the area. The present investigation revealed 52 plants species used for the treatment of different livestock diseases. These plants are used to treat about 10 different types of livestock diseases. Among various classes of traditional uses all across the globe, various types of gastrointestinal disorders are predominant, and a sizeable number of plant species have been discovered to cure such illness across different ethnic communities. Our results are well in agreement with the earlier reports (Davis et al., 1995; Al-Khamees, 1995; Yazicioglu et al., 1996; Sher et al., 2010c). In general, the documented plant species were different from our reported plant species, however, some plants used in Afghanistan and elsewhere were found common (Davis et al., 1995; Goshi, 1995).

It was also observed that some plants had single medicinal uses, while many other had multiple uses. These were invariably used for curing various diseases and for earning a livelihood. The findings of the present study are in line with the studies conducted earlier (Trumble et al., 2004; Saganuwan, 2010; Abdillahi et al., 2010). It is well understood that ethnobotanical studies play a vital role in exploration of plants and human interaction, which in turn help in searching for an alternate source of income and medicine. It is worth specifying that the reported plants were severely grazed by the local livestock in the currently selected study sites. Overgrazing caused the destruction of green parts of plants or such plant parts were removed and damaged due to trampling. It is therefore important to manage the grazing system and encourage the regeneration of medicinal plants.
Table 1. Plants of veterinary importance used in Arab system of medicines in Riyadh Region of Saudi Arabia.

<table>
<thead>
<tr>
<th>No.</th>
<th>Botanical name</th>
<th>Family</th>
<th>Local name</th>
<th>Voucher specimen number</th>
<th>Part used</th>
<th>Habit</th>
<th>Types of animals</th>
<th>Types of disease treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aerva javanica (Burm. F.) Spreng.</td>
<td>Amaranthaceae</td>
<td>Erwa</td>
<td>H-KSU/06-2010-1</td>
<td>Leaves and shoot</td>
<td>Herb</td>
<td>Camels</td>
<td>Fever, UTI</td>
</tr>
<tr>
<td>2</td>
<td>Amaranthus viridis L.</td>
<td>Amaranthaceae</td>
<td>Qutafa</td>
<td>H-KSU/06-2010-2a</td>
<td>Leaves and shoot</td>
<td>Herb</td>
<td>Camels</td>
<td>Stomachic</td>
</tr>
<tr>
<td>3</td>
<td>Anicosciadium lanatum Boiss.</td>
<td>Apiaceae</td>
<td>Lassai</td>
<td>H-KSU/06-2010-40</td>
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### Conclusions

The investigated area host many endemic and endangered species of plants, many of them are of medicinal and economic importance. Indigenous knowledge behind the uses, collection and management of medicinal plant species is rapidly eroding. One reason for this is the lack of awareness among the local community regarding the economic and medicinal importance of medicinal plants. Another factor contributing to the declination of medicinal plants cover and eroding of indigenous knowledge is the inadequacy of the medicinal plants market and lack of government support. This is therefore an issue of national policies and must be addressed.

The approach to improve or restore the ill effects of resources misuse and economic degradation should be in multiple directions, from improving the economic standard to changing the attitudes of the local people should be adopted in future. This type of study may help in better understanding of local forest resources and potential medicinal and aromatic plants. Lack of knowledge regarding the local potential at the national level would eventually lead to the genetic erosion of medicinal plant species and the related indigenous knowledge system.

In order to ensure the management and conservation of medicinal plants, documenting of indigenous knowledge system and its constant and consisting support is essential.

### ACKNOWLEDGEMENT

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Full Length Research Paper

Potential of soil fungi to produce penicillin V acylase and its optimization conditions

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Fifty fungal isolates representing 31 species, related to 8 genera, were isolated from soil and screened for their abilities to produce endo- and exocellular penicillin V acylase enzymes. For endocellular enzyme, eleven isolates (represent 22% of total isolates) exhibited high enzyme activity and 9 isolates (18%) had moderate ability. However, twenty isolates (40%) were low producers and ten isolates (20%) had no ability to produce the enzyme. For exocellular enzyme, four isolates only (representing 8% of total isolates) exhibited high enzyme activity, and fifteen isolates (30%) were found to be moderate. However, twenty two isolates (44%) were low producers and nine isolates (18%) had no activity. The most active fungal isolates were Aspergillus terreus and Penicillium chrysogenum, producing 325 and 280 U/ml, respectively. Maximum activity of penicillin V acylase produced by A. terreus and P. chrysogenum were obtained after 7 and 6 days of incubation, respectively at 35°C and initial pH 6. Dextrin or glucose was the best carbon source for penicillin V acylase produced by A. terreus and P. chrysogenum, respectively. However, peptone was the best nitrogen source. Inoculation of cultures by 3 discs of fungi and incubation of cultures at 180 rpm shaking condition improved the enzyme production.

Key words: Penicillin V acylase, soil fungi, Aspergillus terreus, Penicillium chrysogenum.

INTRODUCTION

Penicillin acylase is one of the most important enzymes applied in the pharmaceutical industry for large scale production of 6-aminopenicillanic acid. This enzyme is the starting material for the manufacture of penicillin derivatives. β-lactam antibiotics, like penicillins and cephalosporins represent one of the major world's biotechnology markets, with annual sales of 15 billion Dollars and about 65% of the total antibiotic market (Chou et al., 2000; Parmar et al., 2000; Elander, 2003; Chandel et al., 2008).

Due to the worldwide demand for semisynthetic penicillins, production of 6-aminopenicillanic acid has been increased up to 7000 tons (Javadvour et al., 2002).

Penicillin G acylase is estimated to be in the range of 10 to 30 million tons (Chandel et al., 2008). Penicillin acylases are microbial enzymes and are found in a wide range of bacteria, actinomycetes, yeasts and filamentous fungi (Suddhakaran and Borkar, 1985a, b). Based on their substrate specificities, they have been classified into three groups: Penicillin V acylase, penicillin G acylase and ampicillin acylase. Penicillin G acylase is present in several bacteria (Arshad and Ahmad, 2000; Abedi et al., 2004; Souza et al., 2005; Arshad et al., 2006; Cheng et al., 2006; Yang et al., 2006; Zuza et al., 2007; Chandel et al., 2008; El Enshasy et al., 2009; Kafshnochi et al., 2010).

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Recently, penicillin G acylase DNA of *Shigella boydii* was isolated, amplified and sequenced. The sequencing gene composed a polypeptide of 846 amino acid residues, and the highest homology was about 96% (Hassan et al., 2009). Penicillin V acylase is produced mainly by moulds and actinomycetes. They are mainly intracellular enzymes and also could produce from genus *Fusarium* (Lowe et al., 1986; Sudhakaran and Shewale, 1993) and actinomycetes *Streptomyces lavendulae* (Torres-Bacete et al., 2000) as extracellular acylases.

The enzymes which hydrolyze the peptide linkage are named penicillin acylase. Acylases are differing in their properties according to the source they come from such as bacteria, yeasts or filamentous fungi (Claridge et al., 1963; Spence and Ramsden, 2007). Fungal acylase hydrolyze phenoxymethyl penicillin more rapidly than benzylpenicillin. This enzyme has been described in many species of actinomycetes, yeasts and filamentous fungi, including genera *Alternaria*, *Aspergillus*, *Botrytis*, *Cephalosporium*, *Cryptococcus*, *Emericellopsis*, *Epichloe*, *Epidermophyton*, *Fusarium*, *Mucor*, *Penicillium*, *Phoma*, *Trichoderma*, *Trichophyton* and *Trichosporon* (Erickson and Bennett, 1965; Cole, 1966; Hamilton-Miller, 1966, 2008; Lowe et al., 1986; Sudhakaran and Shewale, 1993; Jose et al., 2003; Bashir et al., 2008).

The *Fusarium* sp. SKF 235 produced penicillin V acylase intracellularly and extracellularly. The maximum yield of penicillin V acylase was 430 IU/g dry cell wet. This maximum activity was achieved at pH 6.5. Penicillin V acylase from *Fusarium moniliforme* and *Fusarium avenaceum* showed maximum activity at pH values of 8.5 and 7.5, respectively (Vanderhaeghe et al., 1968; Vandamme and Voets, 1972). The optimum pH values for the penicillin V acylase from *Fusarium* sp. SKF 235 is therefore lower than that from other *Fusarium* isolates. This low optimum pH value (6.5) is advantageous for the commercial production of 6-aminopenicillanic acid from penicillin V since the stability of penicillin V in solution is maximum at pH 5.7. On the other hand, pharmacological studies of some plant extracts showed various antibacterial and antifungal activities such as *Peganum harmala* (Asgarpanah and Ramezanloo, 2012), *Viburnum grandiflorum* (Alam et al., 2012) and *Allium cepa* (Nasri et al., 2012). This research was carried out to investigate the incidence of penicillin V acylase enzyme in different genera and species of soil fungi as well as the optimum conditions for the production of this enzyme by the most active fungal isolates.

**MATERIALS AND METHODS**

**Screening of fungi for production of penicillin V acylase enzyme**

Fifty fungal isolates representing 31 species related to 8 genera were recovered from soil and screened for their abilities to produce intracellular and extracellular penicillin V acylase enzymes. These isolates belong to several genera including *Aspergillus* (10 species), *Emericella* (1 species), *Fusarium* (7 species), *Gibberella* (3 species), *Nectria* (1 species), *Paeclomyces* (1 species), *Penicillium* (7 species) and *Trichoderma* (1 species).

**Production medium**

Penicillin V acylase was tested by using the following standard medium which contained the following constituents (g/L): Glucose, 20; peptone 5; yeast extract, 5; K$_2$HPO$_4$, 1; MgSO$_4$.7H$_2$O, 0.2 and penicillin V as inducer 0.3. The pH was adjusted to 5.8 (Bashir et al., 2008).

**Cultivation and culture conditions**

The medium was distributed in Erlenmeyer flasks (250 ml) each flask contained 100 ml of the medium. The flasks were sterilized at 121°C for 20 min. Each flask was inoculated with 10 mm mycelial disc cut out from 5 days fungal colony grown on glucose-Czapek's agar medium. The inoculated flasks were incubated under shaking condition (180 rpm) at 30°C for 7 days. At the end of the incubation period, the content of each flask was filtrated.

**Determination the activity of penicillin V acylase**

Three grams of wet biomass was weighed and suspended in a 6 ml mixture of 0.1 M phosphate buffer (pH = 7), 20% sucrose and 1 mM EDTA (ethylene diamine tetraacetic acid). 0.5 g of glass beads were added and the microtubes were placed in a bead mill at 4°C for 25 min, followed by centrifugation at 13,000 rpm for 5 min in case of intracellular enzyme but in case of extracellular enzyme, the supernatant was used directly after filtration and centrifugation of samples. 6-aminopenicillanic acid was determined by colorimetric method as described by Balasingham et al. (1972). The mixture of 0.25 ml of 0.1 M phosphate buffer (pH = 7) containing 2.5 mg of penicillin V, 0.25 ml of cell suspension (intracellular enzyme) or supernatant (extracellular enzyme) was incubated at 37°C for 30 min, 3 ml of solution made by mixing 2 ml of glacial acetic acid with 1 ml of 0.05 M NaOH was added to the reaction mixture and centrifuged. 0.5 ml of 0.5% P-dimethylaminobenzaldehyde in methanol was added to the supernatant, and the ultra violet (UV) absorbance was measured at 415 nm. One unit of enzyme activity was defined as the quantity of the enzyme which produced 1 μmol of 6-aminopenicillanic acid per 1 min.

**Effect of incubation periods on penicillin V acylase production by *Aspergillus terreus* and *Penicillium chrysogenum***

*A. terreus* and *P. chrysogenum* were found to be the most active penicillin V acylase (endoenzyme) producers. So these fungi were used for further studies. The medium previously described by Bashir et al. (2008) was employed.

**Cultivation and culture conditions**

The influence of different incubation periods (2, 4, 6, 7, 8, 10 and 12 days) on the production of penicillin V acylase and fungal dry weight were measured. The flasks were sterilized at 121°C for 20 min, inoculated with 10 mm disc cut out from 5 days fungal colony grown on glucose-Czapek's agar medium. Two replicates were used for each treatment. Inoculated flasks were incubated under shaking condition (180 rpm) at 30°C. At the end of different incubation periods, fungal mycelium was separated from the growth
Effect of inoculum size on penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum

The influence of inoculum size on the production of penicillin V acylase was tested by inoculating of culture medium with 1 disc, 2 discs and 3 discs (10 mm in diameter) cut out from 5 days colony of the tested fungi grown on glucose-Czapek’s agar medium. The flasks were incubated under shaking condition (180 rpm) at 30°C for 7 days. After the incubation period, penicillin V acylase activity was measured in two replicates according to method described by Balasingham et al. (1972). Fungal dry weight was also determined.

Effect of shaker speed on penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum

This experiment occurred by using 2 volumes of liquid synthetic medium (50 and 100 ml) in 250 ml Erlenmeyer flasks. The two fungi were grown in Erlenmeyer flasks (250 ml) containing 100 ml liquid medium. The flasks were divided into 3 groups: The first group placed on rotary shaker at 160 rpm, the second placed on rotary shaker at 180 rpm and the third group placed on rotary shaker at 200 rpm for 7 days at 30°C. After the incubation period, the activity of the enzyme was determined according to method described by Balasingham et al. (1972). Fungal dry weight was also determined.

Effect of medium volume on penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum

This experiment occurred by using 2 volumes of liquid synthetic medium (50 and 100 ml) in 250 ml Erlenmeyer flasks. The two fungi were grown in Erlenmeyer flasks (250 ml) containing 100 ml liquid medium. The flasks were divided into 3 groups: The first group placed on rotary shaker at 160 rpm, the second placed on rotary shaker at 180 rpm and the third group placed on rotary shaker at 200 rpm for 7 days at 30°C. After the incubation period, the activity of the enzyme was determined according to method described by Balasingham et al. (1972). Fungal dry weight was also determined.

RESULTS AND DISCUSSION

Screening of fungi for their abilities to produce penicillin V acylase enzyme

Screening of fifty fungal isolates for production of endocellular penicillin V acylase showed that eleven isolates represented 22% of total isolates which showed high enzyme activity. Nine isolates contributed 18% of total isolates found to be moderate activity. Twenty isolates represented 40% of total isolates which were low producers of penicillin V acylase enzyme. Ten isolates represented 20% of total isolates which had no enzyme activity (Table 1).

For exocellular penicillin V acylase, four isolates (represent 8% of total isolates) only showed high enzyme activity and fifteen isolates (30%) were moderate. However, twenty two isolates (44%) were low producers of the enzyme. Nine isolates (18%) had no activity. A. terreus and P. chrysogenum were the most active producer isolates of fungi for penicillin V acylase. These fungi produced 325 and 280 U/ml, respectively (Table 1). Bashir et al. (2008) screened 12 different strains of fungi for their abilities to produce penicillin G acylase enzyme under submerged condition. They showed that A. niger had the highest enzyme activity.
Table 1. Screening of fungi for their abilities to produce endo- and exoenzyme penicillin V acylase.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Enzyme concentration</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endoenzyme</td>
<td>Exoenzyme</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>22.85 L</td>
<td>32.85 L</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>-</td>
<td>6.42 L</td>
</tr>
<tr>
<td>A. glaucus</td>
<td>180.00 H</td>
<td>-</td>
</tr>
<tr>
<td>A. niger</td>
<td>11.42 L</td>
<td>127.14 M</td>
</tr>
<tr>
<td>A. ochraceus</td>
<td>7.14 L</td>
<td>115.71 M</td>
</tr>
<tr>
<td>A. sydowii</td>
<td>141.42 M</td>
<td>-</td>
</tr>
<tr>
<td>A. tamarii</td>
<td>214.28 H</td>
<td>-</td>
</tr>
<tr>
<td>A. terreus</td>
<td>325.00 H</td>
<td>3.57 L</td>
</tr>
<tr>
<td>A. ustus</td>
<td>84.28 M</td>
<td>114.28 M</td>
</tr>
<tr>
<td>A. versicolor</td>
<td>24.28 L</td>
<td>5.71 L</td>
</tr>
<tr>
<td>Emericella nidulans</td>
<td>179.20 H</td>
<td>14.28 L</td>
</tr>
<tr>
<td>Fusarium chlamydosporum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F. compactum</td>
<td>5.71 L</td>
<td>14.28 L</td>
</tr>
<tr>
<td>F. heterosporum</td>
<td>84.28 M</td>
<td>114.20 M</td>
</tr>
<tr>
<td>F. mersmipoides</td>
<td>197.14 H</td>
<td>45.71 L</td>
</tr>
<tr>
<td>F. oxysporum</td>
<td>7.14 L</td>
<td>37.85 L</td>
</tr>
<tr>
<td>F. sambucinum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F. tricinum</td>
<td>15.71 L</td>
<td>51.42 M</td>
</tr>
<tr>
<td>Gibberella avenacea</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G. fujikuroi</td>
<td>-</td>
<td>37.14 L</td>
</tr>
<tr>
<td>G. intricans</td>
<td>7.85 L</td>
<td>83.57 M</td>
</tr>
<tr>
<td>Nectria haematococca</td>
<td>8.57 L</td>
<td>91.42 M</td>
</tr>
<tr>
<td>Paeclomyces lilacinus</td>
<td>10.00 L</td>
<td>107.14 M</td>
</tr>
<tr>
<td>Penicillum aurantiogriseum</td>
<td>152.85 H</td>
<td>5.00 L</td>
</tr>
<tr>
<td>P. brevicompectum</td>
<td>126.42 M</td>
<td>70.71 M</td>
</tr>
<tr>
<td>P. chrysogenum</td>
<td>152.85 H</td>
<td>236.42 H</td>
</tr>
<tr>
<td>P. duclauxii</td>
<td>88.57 M</td>
<td>225.00 H</td>
</tr>
<tr>
<td>P. funiculosum</td>
<td>280.00 H</td>
<td>22.14 L</td>
</tr>
<tr>
<td>P. funiculosum</td>
<td>80.00 M</td>
<td>65.71 M</td>
</tr>
<tr>
<td>P. funiculosum</td>
<td>28.57 L</td>
<td>44.28 L</td>
</tr>
</tbody>
</table>
Table 1. Contd.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme activity</th>
<th>Dry weight (mg/100 ml)</th>
<th>Enzyme activity</th>
<th>Dry weight (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. purpurogenum</em></td>
<td>101.42 M</td>
<td>35.71 L</td>
<td>2924.41</td>
<td>1029.68</td>
</tr>
<tr>
<td><em>P. roqueforti</em></td>
<td>25.00 L</td>
<td>2.85 L</td>
<td>720.86</td>
<td>82.17</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>- 2.14 L</td>
<td>-</td>
<td>61.70</td>
<td></td>
</tr>
</tbody>
</table>

High (H) > 150, Moderate (M) = 50 to 150, Low (L) < 50, - = no enzyme detected.

Table 2. Effect of incubation periods on growth and penicillin V acylase production by *Aspergillus terreus* and *Penicillium chrysogenum*.

<table>
<thead>
<tr>
<th>Incubation period (days)</th>
<th>Aspergillus terreus</th>
<th>Penicillium chrysogenum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
<td>Enzyme activity</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2883.46</td>
<td>383.44</td>
</tr>
<tr>
<td>6</td>
<td>8650.40</td>
<td>403.21</td>
</tr>
<tr>
<td>7</td>
<td>9062.15</td>
<td>432.10</td>
</tr>
<tr>
<td>8</td>
<td>7826.30</td>
<td>391.20</td>
</tr>
<tr>
<td>10</td>
<td>4119.03</td>
<td>382.80</td>
</tr>
<tr>
<td>12</td>
<td>2471.41</td>
<td>371.70</td>
</tr>
</tbody>
</table>

Effect of incubation periods on growth and penicillin V acylase by *Aspergillus terreus* and *Penicillium chrysogenum*

*A. terreus* and *P. chrysogenum* exhibited maximum penicillin V acylase production at 7 and 6 days of incubation, respectively. These fungi had enzyme activity of 9062.15 and 7826.3 U/ml, respectively. At 8 days of incubation, the two tested fungi produced relatively considerable amounts of penicillin V acylase enzyme, however decreasing of incubation period to 2 or 4 days had negative effect on the enzyme production by the two tested fungi. Increasing of incubation period to 10 or 12 days decreased the production of penicillin V acylase enzyme by *A. terreus* and *P. chrysogenum*. The results recorded for the effect of incubation time on mycelial growth of *A. terreus* and *P. chrysogenum* was nearly similar to those reported for penicillin V acylase enzyme (Table 2). In this respect, maximum production of penicillin G acylase of *A. niger* was recorded between 20 to 24 h of incubation under shaking conditions (Bashir et al., 2008).

Effect of temperatures on fungal growth and penicillin V acylase production by *A. terreus* and *P. chrysogenum*

Temperature greatly influenced the production of penicillin V acylase by *A. terreus* and *P. chrysogenum*. Maximum production of the enzyme was recorded at 35°C by the two tested fungi producing 9062.15 and 7785.36 U/ml, respectively. Decreasing of incubation temperature below 35°C decreased the enzyme production. Decreasing of the incubation temperature to 15°C prevented the secretion of the enzyme by the two fungi. Also increasing of incubation temperature to 40°C decreased the production of penicillin V acylase. The results obtained for the effect of incubation temperature on mycelial growth of *A. terreus* and *P. chrysogenum* was variable with different incubation temperatures, with maximum growth at 25 and 35°C, respectively (Table 3). The optimum temperature for maximal penicillin G acylase enzyme of *A. fumigatus* and *Mucor gryseoclanum* was at 40°C (Jose et al., 2003). Abedi et al. (2004) showed that the optimum temperature for penicillin G acylase synthesis activity was at 25°C. Souza et al. (2005) reported that penicillin G acylase produced by *Bacillus megaterium* showed maximum hydrolysis rate at 37°C. Zuza et al. (2007) found that the optimal temperature of *Escherichia coli* penicillin acylase activity were 27.5 and 31.5°C for free and immobilized enzyme, respectively.

Effect of pH values on growth and penicillin V acylase production by *A. terreus* and *P. chrysogenum*

The pH value was greatly affected the production of penicillin V acylase by *A. terreus* and *P. chrysogenum*. Maximum production of the enzyme was recorded at pH 6 by the two fungi, producing 9062.15 and 7002.49 U/ml, respectively. Decreasing of pH value to pH 4 exhibited an inhibitive effect on penicillin V acylase production. Also, increasing of pH value to 7, 8 or 10 decreased the
Table 3. Effect of temperatures on growth and penicillin V acylase production by *Aspergillus terreus* and *Penicillium chrysogenum*.

<table>
<thead>
<tr>
<th>Temperature</th>
<th><em>Aspergillus terreus</em></th>
<th><em>Penicillium chrysogenum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>4984.07</td>
<td>402.03</td>
</tr>
<tr>
<td>25</td>
<td>7950.00</td>
<td>419.09</td>
</tr>
<tr>
<td>30</td>
<td>8609.16</td>
<td>413.35</td>
</tr>
<tr>
<td>35</td>
<td>9062.15</td>
<td>390.67</td>
</tr>
<tr>
<td>40</td>
<td>3624.80</td>
<td>302.15</td>
</tr>
</tbody>
</table>

Table 4. Effect of pH values on growth and penicillin V acylase production by *Aspergillus terreus* and *Penicillium chrysogenum*.

<table>
<thead>
<tr>
<th>pH value</th>
<th><em>Aspergillus terreus</em></th>
<th><em>Penicillium chrysogenum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>4119.03</td>
<td>301.50</td>
</tr>
<tr>
<td>6</td>
<td>9062.15</td>
<td>493.51</td>
</tr>
<tr>
<td>7</td>
<td>8238.35</td>
<td>482.10</td>
</tr>
<tr>
<td>8</td>
<td>7414.54</td>
<td>372.10</td>
</tr>
<tr>
<td>10</td>
<td>2471.41</td>
<td>294.31</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

enzyme production by the two tested fungi. No enzyme production was recorded at pH 2 and 12 by the two fungal species. Data recorded for the effect of pH value on mycelial growth of *A. terreus* and *P. chrysogenum* was nearly similar to those reported for penicillin V acylase enzyme (Table 4). Javadpour et al. (2002) reported that the optimal pH of penicillin acylase produced by *E. coli* was determined to be 8. The optimum pH range for dialyzed preparations of penicillin G acylase produced by *A. fumigatus* and *M. gryseoclanum* was 7 to 8 and 7.5 to 8.5, respectively (Jose et al., 2003). Abedi et al. 2004 showed that the optimum pH for penicillin G acylase synthesis activity was 6. Souza et al. (2005) reported that penicillin G acylase produced by *Bacillus megaterium* showed maximum hydrolysis rate at pH 8. Zuza et al. (2007) reported that the optimal pH of *E. coli* penicillin acylase activity was 8.5 for both free and immobilized enzyme.

**Effect of different carbon sources on growth and penicillin V acylase production by *A. terreus* and *P. chrysogenum***

Carbon source is one of the most essential components in the microbial medium. The effect of different carbon sources on growth and penicillin V acylase production by *A. terreus* and *P. chrysogenum* was variable. Glucose and dextrin were the most favorable carbon sources for penicillin V acylase production by *P. chrysogenum* and *A. terreus*, respectively. Maximum production of the enzyme was achieved by incorporation of glucose and dextrin in the culture medium for enzyme production, producing 8897.51 and 7702.89 U/ml for *A. terreus* and *P. chrysogenum*, respectively. Addition of other carbon sources in the fungal cultures decreased the enzyme production by the two fungal species. Mycelial growth of *A. terreus* and *P. chrysogenum* was not greatly affected by the addition of different carbon sources to the culture medium for fungi (Table 5). Among 7 carbon sources tested for penicillin G acylase, 0.4% lactose was the best for mycelial growth and enzyme production of *A. niger* (Bashir et al., 2008). Other carbon sources such as glucose, sorbitol glycerol, sucrose, PAA (Phenyl Acetic Acid) and tryptone increased the activity of Penicillin G acylase by *E. coli* and *Bacillus megaterium* (Chandel et al., 2008).

**Effect of different nitrogen sources on growth and penicillin V acylase production by *A. terreus* and *P. chrysogenum***

Nitrogen source is one of the most essential components in the microbial medium because the nitrogen element...
Table 5. Effect of different carbon sources on growth and penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum.

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Aspergillus terreus</th>
<th>Penicillium chrysogenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
</tr>
<tr>
<td>Glucose</td>
<td>5931.57</td>
<td>384.00</td>
</tr>
<tr>
<td>Fructose</td>
<td>2965.64</td>
<td>395.21</td>
</tr>
<tr>
<td>Maltose</td>
<td>6590.73</td>
<td>394.47</td>
</tr>
<tr>
<td>Dextrin</td>
<td>8897.51</td>
<td>381.45</td>
</tr>
<tr>
<td>Lactose</td>
<td>2965.64</td>
<td>394.25</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3295.22</td>
<td>387.50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>1647.61</td>
<td>380.57</td>
</tr>
<tr>
<td>Starch</td>
<td>1977.19</td>
<td>385.90</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2636.06</td>
<td>393.73</td>
</tr>
</tbody>
</table>

Table 6. Effect of different nitrogen sources on growth and penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum.

<table>
<thead>
<tr>
<th>Nitrogen sources</th>
<th>Aspergillus terreus</th>
<th>Penicillium chrysogenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>8238.35</td>
<td>357.91</td>
</tr>
<tr>
<td>(NH₄)₂H₂PO₄</td>
<td>4942.83</td>
<td>361.37</td>
</tr>
<tr>
<td>KNO₃</td>
<td>5931.57</td>
<td>369.32</td>
</tr>
<tr>
<td>CH₃COONH₄</td>
<td>5602.03</td>
<td>364.46</td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
<td>3954.38</td>
<td>373.53</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>6261.15</td>
<td>359.01</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>5931.57</td>
<td>366.03</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>7249.69</td>
<td>357.77</td>
</tr>
<tr>
<td>Peptone</td>
<td>9103.39</td>
<td>360.51</td>
</tr>
</tbody>
</table>

participates in the formation of amino and nucleic acids and protein in microbial cells. The effect of different nitrogen sources on fungal growth and penicillin V acylase production by A. terreus and P. chrysogenum varied. Among nine nitrogen sources studied, peptone was the most suitable nitrogen source for penicillin V acylase production.

Maximum production of the enzyme was recorded by the incorporation of peptone in the culture medium for enzyme production, producing 9103.39 and 7826.30 U/ml by A. terreus and P. chrysogenum, respectively. Addition of ammonium sulphate and ammonium chloride to the fungal cultures exhibited considerable amount of penicillin V acylase.

However, the other nitrogen sources decreased the enzyme production. Mycelial growth of A. terreus and P. chrysogenum was not greatly affected by the addition of different nitrogen sources to the culture medium for fungi (Table 6). Chandel et al. (2008) reported that maximum Penicillin G acylase activity was obtained using casein hydrolysate supplemented with 0.6 L of alcalase and cheese whey.

Effect of inoculum size on growth and penicillin V acylase production by A. terreus and P. chrysogenum

The inoculum size influenced the production of penicillin V acylase by A. terreus and P. chrysogenum. Maximum yield of penicillin V acylase was achieved by inoculating of liquid synthetic medium with 3 discs (10 mm diameter) of fungi. A. terreus produced 9759.38, corresponding to 7785.36 U/ml for P. chrysogenum. Decreasing of the inoculum size to 2 or 1 disc decreased the production of penicillin V acylase enzyme by the two fungal species. Mycelial growth of A. terreus and P. chrysogenum did not greatly affected by inoculation size of fungi (Table 7). Spore concentration 1.5 × 10⁷ spores/ml and germination during 24 and 72 h showed maximum
Table 7. Effect of inoculum size on growth and penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum.

<table>
<thead>
<tr>
<th>Inoculum size</th>
<th>Aspergillus terreus</th>
<th>Penicillium chrysogenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
</tr>
<tr>
<td>1 disc</td>
<td>6653.88</td>
<td>384.43</td>
</tr>
<tr>
<td>2 discs</td>
<td>6243.57</td>
<td>389.04</td>
</tr>
<tr>
<td>3 discs</td>
<td>9759.38</td>
<td>391.70</td>
</tr>
</tbody>
</table>

Table 8. Effect of medium volume on growth and penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum.

<table>
<thead>
<tr>
<th>medium volume (ml)</th>
<th>Aspergillus terreus</th>
<th>Penicillium chrysogenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
</tr>
<tr>
<td>100</td>
<td>9981.11</td>
<td>360.40</td>
</tr>
<tr>
<td>50</td>
<td>4077.79</td>
<td>356.04</td>
</tr>
</tbody>
</table>

Table 9. Effect of shaker speed on growth and penicillin V acylase production by Aspergillus terreus and Penicillium chrysogenum.

<table>
<thead>
<tr>
<th>Shaker speed (rpm)</th>
<th>Aspergillus terreus</th>
<th>Penicillium chrysogenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity</td>
<td>Dry weight (mg/100 ml)</td>
</tr>
<tr>
<td>160</td>
<td>8032.47</td>
<td>351.55</td>
</tr>
<tr>
<td>180</td>
<td>9479.39</td>
<td>388.10</td>
</tr>
<tr>
<td>200</td>
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<td>379.15</td>
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</tbody>
</table>

Penicillin G acylase activity by B. megaterium (Chandel et al., 2008).

Effect of shaker speed on growth and penicillin V acylase production by A. terreus and P. chrysogenum

Speed of shaker had an effect on growth and penicillin V acylase production by A. terreus and P. chrysogenum. A. terreus and P. chrysogenum showed maximum penicillin V acylase production (9479.39 and 7723.36 U/ml, respectively) at 180 rpm. Mycelial growth of A. terreus and P. chrysogenum was not greatly affected by incubation shaking of fungal cultures (Table 8). Recently, Saleem et al. (2012) reported that the endocellular D-amino acid oxidase enzyme produced by Fusarium heterosporum and Nectria haematococca showed maximum activity in cultures incubated at 160 rpm shaking condition.

Effect of medium volume on growth and penicillin V acylase production by A. terreus and P. chrysogenum

The effect of medium volume on growth and penicillin V acylase production by A. terreus and P. chrysogenum was studied by using different volumes of cultivation medium. A. terreus and P. chrysogenum produced higher amounts of penicillin V acylase (9981.11 and 8672.31 U/ml, respectively) in flask containing 100 ml of medium. Decreasing of the amount of medium to 50 ml decreased the enzyme production by nearly half amount (4077.79 and 4736.95 U/ml, respectively). Mycelial growth of A. terreus and P. chrysogenum was not greatly affected by the amount of medium (Table 9). Saleem et al. (2012) found that D-amino acid oxidase enzyme and mycelial growth of F. heterosporum and Nectria haematococca were higher in flasks containing 100 ml of medium than those containing 50 ml of medium by nearly half amount.

Conclusion

This article would contribute to finding new sources of penicillin acylases by fungi and increase the production of these enzymes by optimizing cultural conditions which will lead to the production of some antibiotics on a large scale and development of some antibiotic manufacture.

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Spectrophotometric study of the anti-caseinolytic activity of root extracts of *Teclea nobilis* and *Vepris zambesiaca* on *Bitis arietans* venom

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The study focused on the inhibition of the caseinolytic activity of *Bitis arietans* venom by aqueous methanol crude root extracts of *Teclea nobilis* and *Vepris zambesiaca*. Snake venom samples were collected from Snake World near Harare, and the medicinal plants were obtained from Mash Central Province, Zimbabwe. Data on the kinetics of the digestion of casein by the *B. arietans* venom and the inhibition of the caseinolytic activity of the venom were generated spectrophotometrically. Lineweaver-Burk plots to explore the kinetics of the digestion of casein by *B. arietans* venom gave $v_{\text{max}}$ and $K_M$ values of $8.33 \times 10^3$ mol dm$^{-3}$ s$^{-1}$ and $7.35 \times 10^2$ mol dm$^{-3}$, respectively. Maximum inhibition of caseinolytic activity of the *B. arietans* venom of 70 and 60% was observed when incubation was performed in the presence of 6.25 and 8.50 µg/ml of *T. nobilis* and *V. Zambesiaca*, respectively. The inhibitory effect of the extracts was correlated to the levels of flavonoids, flavonols and phenolics. The results demonstrate that *T. nobilis* and *V. zambesiaca* have great potential as medicinal plants and are possible candidates for new therapeutic agents in the treatment of snake bite envenomations.

Key words: *Bitis arietans*, inhibition, caseinolytic effect, *Teclea nobilis*, *Vepris zambesiaca*.

INTRODUCTION

Snakebite envenomations continue to be a threat to public health in some parts of the world. At least 1,841,000 snakebites resulting in about 94,000 deaths are recorded annually (Chippaux et al., 1991; Pugh and Theakston, 1980). In India alone, 35,000 to 50,000 die of snakebites annually (Morais and Massaldi, 2009). In Zimbabwe, about 270 cases of snakebites are recorded annually with a mortality rate of about 2% (Nhachi and Kasilo, 1994), and a significant number of victims are not reported. Common poisonous snakes in Zimbabwe belong to four families; Colubrids (Boomslang), Vipers (Puff Adder), Elapids (Mambas and Cobras) and Atractaspids (Bibron Stiletto snake) (Muguti et al., 1994).

Snake venom is a complex mixture containing several enzymes, peptides and non-protein components (Chippaux et al., 1991; Rucavado et al., 2004; Pithayanukul et al., 2009). The physiopathological effects of venom results from synergic action of the composite mixture (Currier et al., 2010). The amount of snake venom component depends on the snake species, diet, season and geographical regions (Rodrigues et al., 1998; Currier et al., 2010). *Bitis arietans* venom is mainly haemorrhagic and myotoxic which result from the proteinases and proteases action, mainly metalloproteinases and serine proteases. Haemorrhagic and proteases activity are the main cause of local tissue damage. *In vitro* assay using casein and fibrinogen are some of the methods used to measure haemorrhagic and proteolytic activities (Biondo et al., 2003). The conventional antivenoms are expensive and do not combat local tissue damage except Batimastat. Although there are several snake venom components,
proteinases mainly cause local tissue damage which has been shown to be of medical importance. Anti-venom immunotherapy has been the only specific treatment against snake envenomations (Arce et al., 2003; Moraes et al., 2003; Gutiérrez et al., 1990). However, there are various side effects of sera antivenoms such as bronchospasms, anaphylactic shock, serum sickness and pyrogen reaction (Lomente et al., 2008; Feofanov et al., 2005; Meenatchisundaram et al., 2009; Wong et al., 2010). Moreover immunotherapy is relatively inefficient to neutral venom-induced local tissue damage (Gutiérrez et al., 2006; Gibbs and Mackessy, 2009), and antivenoms are not always readily available in some regions of Africa and Asia (Chippaux et al., 1998; Rodrigues et al., 1998). Therefore the search for novel venom inhibitors has expanded to include the possibility of using plants that may neutralize relevant toxins in the venoms and which may be readily available with minimum side effects (Mors et al., 2000; Borges et al., 2001; Biondo et al., 2003; Alam and Gomes, 2003; Januário et al., 2004; Oliveira et al., 2005).

The use of medicinal plants play a significant role to cover the basic health needs not only in the developing countries, but also in developed countries (Selim et al., 2013). Herbs have been a major source of drug formulations, for instance quinine and cough syrups, were developed from plants. Medicinal plants contain many compounds, such as polyphenols (phenolics), flavonoids, flavonols, alkaloids, saponins and steroids which are among the other phytochemical compounds that have been shown to exhibit therapeutic activity, for example antivenom and anti-microbial properties (Mors et al., 2000; Dey and De, 2012; Selim et al., 2013; Barkatullah et al., 2013). The mechanism of action varies from simple chelation of central metal ions, anti-oxidant scavenging to structural modification of the antigens (Mors et al., 2000).

In some regions of Zimbabwe, venomous snakebites have been traditionally treated with infusions of herbs. However, a survey of literature shows that no studies are documented for the inhibitory activities of locally available plants. The aim of this study was to investigate the inhibitory effect of aqueous methanol crude root extracts of two locally available herbs traditionally used in treatment of snakebites, *T. nobilis* and *V. zambesiaca*, on the in vitro ability of *B. arietans* venom to breakdown casein (its caseinolytic effect). Determining the efficacy of extracts from these plants to neutralize the snake venom resources pave way to possible further studies on the development of anti-snake therapy from locally available resources.

**MATERIALS AND METHODS**

The two plants, *T. nobilis* and *V. zambesiaca*, were collected in Shamva, Northen Zimbabwe and identified at National Herbarium. The plant roots were dug, cleaned and carried to Bindura University Laboratory in previously cleaned polythene bags.

The plant root materials were air dried in a shed for eight weeks to ensure complete dryness (Meenatchisundaram et al., 2009; Biondo et al., 2003). The plant root materials were ground into powder. The ground plant root materials were kept in previously cleaned capped-plastic containers (Alam and Gomes, 2003). The *B. arietans* venom was donated by Snake World in Norton (Located about 15 km west of Harare). The venom was lyophilized and was stored at -20°C. When used in the analysis, the freeze-dried venom was dissolved in phosphate buffer saline (PBS) pH 7.4 to a required concentration. Casein, an analytical grade, was purchased from Sigma-Aldrich.

**Determination of total phytochemicals in the root extracts**

**Flavonoids**

A volume of 0.5 ml of 2% AlCl₃-ethanol solution was added to 0.5 ml of plant extract. After one hour at room temperature, the absorbance was measured at 420 nm (Wolfe et al., 2003). A yellow colour observed was an indication of the presence of flavonoids. The total flavonoids content was evaluated as mg/g quercetin equivalent and this was one-way obtained from the calibration curve (Kessler et al., 2003).

**Flavonols**

A volume of 2 ml of plant extract was added to 2 ml of 2% AlCl₃-ethanol solution and 3 ml of 50 g/l sodium acetate solution. As described by Kumaran and Kumaran (2007), the absorbance was measured at 440 nm after 2½ h at 20°C. The total flavonols were determined from the calibration curve and expressed as mg/g quercetin.

**Phenolics**

Total phenols were determined by the Folin-Ciocalteu reagent method (Kumaran and Karunakaran, 2007). The plant extract (0.5 m of 1:10 g ml⁻¹) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined spectrophotometrically at 765 nm as described by Adedogo et al. (2008).

**Caseinolytic and anti-caseinolytic activity**

A 200 ml casein stock solution of concentration 0.59 g/ml was prepared using the method by Kunitz (1947) and diluted to 1% working solution. A volume of 0.5 ml of crude venom of concentration 0.56 µg/ml in phosphate buffer saline pH 7.4 was added to 1 ml of 1% casein. The stock solution was divided into aliquots of 4 ml each. A set of 40 test tubes containing same concentrations were incubated at 37 °C. The caseinolytic activity was determined by spectrophotometric scanning of the composite mixture of one test tube after the other, at 1 min intervals. Therefore, one test tube was representing a 1 min change in the concentration of casein as a result of the venom action. A set of control experiments with the inhibitor and/or solvents (buffer/ethanol) were performed simultaneously. The percent of inhibition was calculated as:

\[ I(\%) = 1 \frac{A_{\text{max}} - A_{\text{sum}}}{A_{\text{max}} - A_{\text{min}}} \]

Where \(A_{\text{max}}\) is the absorbance of casein not exposed to venom, \(A_{\text{min}}\) is the absorbance of casein exposed to venom.
is the absorbance of casein exposed to venom plus an equal volume of buffer, and $A_{\text{sum}}$ is the absorbance of casein exposed to venom plus an equal volume of sample containing inhibitor (Girish and Kemparaju, 2005). In each case, the test-tubes were incubated at 37°C and scanned after every 1 min using a Shimadzu UV1601 spectrophotometer.

**RESULTS**

The absorbance of casein shown is shown in Figure 1. Casein was found to be absorbed at 288.5 nm using a Shimadzu UV1601 Spectrophotometer. Caseinolytic activity was examined at this wavelength. Figure 2 shows caseinolytic activity of the venom of *B. arietans*. It can be observed that increasing the venom concentration results in an increase in the rate of caseinolytic activity. The graph helps to determine the minimum venom concentration that brings about a 50% ($LD_{50}$) and 100% ($LD_{100}$) caseinolytic activity. Figures 3 and 4 showed that the kinetics followed a first-order mechanism. Inhibition activity (Figure 5) of the crude plant extracts was correlated to total flavonoid, flavonol and phenolic content in *T. nobilis* and *V. Zambesiaca*, and the linear regression parameters are shown in Table 1.

**DISCUSSION**

From *in vitro* tests, caseinolytic activity was determined as the ratio of the absorbance of casein relative to the absorbance of the venom-casein mixture. Figures 1 and 2 show the spectrum of casein and the variation of the caseinolytic activity with venom concentration, respectively. The minimum venom dose that resulted in 100% caseinolytic activity was determined to be 0.56 µg/ml. Plots used to obtain kinetic parameters are shown in Figures 3 and 4, and these were obtained using the Lineweaver-Burk equation:

\[
\frac{1}{v} = \frac{K_M}{V_{max}} \frac{[S]}{V_{max}} + \frac{1}{V_{max}}
\]

Where $v$ is the reaction velocity, $K_M$ is the Michaelis-Menten constant, $V_{max}$ is the maximum reaction velocity, and $[S]$ is the substrate concentration. Values of $K_M$ and $V_{max}$ were obtained as $8.33 \times 10^{-3}$ mol dm$^{-3}$ s$^{-1}$ and $8.33 \times 10^6$ mol dm$^{-3}$, respectively. These results tend to suggest that the digestion of casein by *B. arietans* venom was characterised by fast reaction kinetics.

Caseinolytic activity induced by the crude *B. arietans* venom was inhibited when the venom was incubated with *T. nobilis* and *V. zambesiaca* root extracts at different concentrations (Figure 5). When compared to the inhibitory effect of quercetin as a typical bioactive flavonoid, the plant extracts exhibited a greater inhibitory
effect on the action of the snake venom. Incubating the venom-casein mixture after a dosage of 10.5 µg/ml quercetin resulted in a 50% inhibition, whereas a 70 and 60% inhibition were observed for a 6.25 and 8.50 µg/ml dosage with *T. nobilis* and *V. zambesiaca* extracts, respectively.

The total flavonoid, flavonol and phenolic content of the herbs were higher in *T. nobilis* than in *V. zambesiaca*, as shown in Table 1. These findings tend to suggest that the higher inhibitory effect observed for *T. nobilis* could be explained in terms of the presence of higher levels of bioactive compounds. The mechanisms of action of flavonols, flavonoids and phenolic compounds are through scavenging or chelating process (Girish and Kemparaju, 2005). Such compounds participate in the chelation of the metal atom (zinc) present at the catalytic center of metalloproteinases. Components of the plant extract may occupy sites in the venom, preventing binding of the substrate to the enzymes, and this may take place through covalent or non-covalent bonding (Biondo et al., 2003). However, it suffices to mention that there is still a greater possibility of other bioactive compounds not quantified in this study playing a significant role in the inhibition process.

Substances identified in plants reputed to neutralize the effects of snake venoms, spanning a wide range of molecules, include phenolics, hydroxybenzoic acids, cinnamic acid derivatives, curcuminoids, flavonoids, and tannis, among others (Mors et al., 2000). Additional work to characterise the active phytochemical compounds including those classified under flavonoids, flavonols and phenolics present in *T. nobilis* than in *V. zambesiaca* is
This work was funded by the Research Board.

continued search for effective therapy on the local effects of snakebites.

of some compounds correlated with inhibition of crude venoms showed that the higher inhibitory

effect observed for T. nobilis could be related to the higher composition of flavonoids, flavonols and phenolics. This study therefore provides useful initial findings which can be exploited in the continued search for effective therapy on the local effects of snakebites.

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