Asian Journal of Chemistry; Vol. 26, No. 2 (2014), 499-503

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.15596

# Toxicological Analysis of Ricin in Medicinal Castor Oil with Evaluation of Health Hazards

S. RIAZ and M.A. FARRUKH*

Department of Chemistry, Government College University, Lahore 54000, Pakistan

*Corresponding author: Tel: +92 42 111000010; Ext: 263; Email: akhyar100@gmail.com

*Received*: 6 April 2013; *Accepted*: 18 August 2013; *Published online*: 15 January 2014; AJC-14575

Ricin is a highly toxic, naturally occurring protein in castor bean plant and used as potential bio and chemical warfare agent, through which people can easily be affected by its all routes of exposure. Sixteen different medicinal castor oil samples were checked for contamination with ricin toxin. The classical and gel filtration methods for extraction of purified ricin were adopted. Confirmation of ricin was carried out by gel electrophoresis (SDS-PAGE). FTIR analysis was performed for specific ricin structure determination. Quantification of ricin present in castor oil samples was carried out by observing absorption at 279 nm on UV-visible spectrophotometer. The maximum amount of ricin found in medicinal castor oil samples is 0.713 mol $L^{-1}$ (0.43 mg/10 mL). The possibility of finding ricin in castor oil may be correlated with adverse effects of medicinal castor oil intake. Minimum inhibition concentration of ricin was inferred against *E. coli* and *Staphylococcus* bacterial strains, by agar dilution method. While no effect of ricin was found on *Staphylococcus* strain.

**Keywords: Forensic science, Ricin, Toxicology, Gel electrophoresis, Ultra violet-visible spectra.**

## INTRODUCTION

Ricin is found in castor bean plant (*Ricinus Communis*) of the *Euphorbeace* family of plant kingdom. Ricin contributes 1-5 % of total dry weight of the castor bean and this variability was studied on seeds of different species of castor bean plant[1]. Castor bean contains 40-60 % of castor oil by its weight. Castor beans are also widely differs in their moisture content, in size and weight on the cultivation basis (region, climate conditions and harvesting time for bean maturity)[2].

Ricin is a unique proteinaceous toxin that listed as mid spectrum agent, *i.e.* biological warfare agent and chemical warfare convention. Ricin is a water soluble protein. In solid form, it may be crystalline or white powder. Ricin is highly stable at wide range of pH and at high temperature[3,4]. Continuous boiling at 80 °C required to denature ricin in solution form for approximately 1 h[4]. While in solid or crude form ricin denaturation required even higher temperature for long time duration.

Ricin is a heterodimeric globular protein having molecular weight of 60-66 kDa. With two linked subunits, one of 32-kDa ricin toxin A chain (RTA) that works as ribosomal inactivating protein type II (RIP II; RTA-S-S-RTB) and another of 34-kDa ricin toxin B chain (RTB) which is galactose/N-acetylgalactosamine-binding lectin. Both ricin chains are linked through a disulfide bond which located between the 259 residue on ricin A chain and 4 residue on ricin B chain[5]. Variability found in molecular weight of ricin is attributed to the varying degree of glycosylation of both ricin chains[6]. Amino acid count for mature ricin A chain and ricin B chain is 267 and 262, respectively. Ricin toxin A (RTA) has N-glycosylated activity and it catalyzes the excision of an adenine from the 28S ribosome of eukaryotic RNA[7]. Polypeptide structure of RTA has 50 % α-helice and β-sheet structure with three structural domains on active site[8]. High *in vivo* toxicity is due to RTB that attach to the eukaryotic cell surface by galactose binding site and allow receptor mediated endocytosis of RTA to enter the cell[9]. On internalization of ricin A and B chain into cell, the reduction potential inside the cell allows the release of ricin A chain which then inhibits the protein synthesis[10].

**Toxico-kinetics of ricin:** Ricin may be the most potent toxin among plant toxins. It is determined that a single ricin A chain (RTA) can inactivate 1500 ribosome per minutes and a single molecule of ricin is able to kill an eukaryotic cell[11]. Lethal dose ($LD_{50}$) of ricin in human depends on the route of exposure, in the order of injection 1-1.75 μg/kg > inhalation 21-42 μg/kg > digestion 1-20 mg/kg[12] that is concluded with respect to the toxicity of ricin in mice. Ricin causes damage on exposure by all the routes when it enters the body but damage is largely depends on the amount of ricin.

**Toxico-analysis of ricin:** Ricin is the most commonly used agent for biocrimes. Different prototypes were developed for rapid detection of ricin by use of different analytical methods, probably for the safety measures of comman man.

Detection of ricin in tampered food samples by FT-NIR[13] and by liquid chromatography LC-MS-MS[14] has been employed. While mass spectrometric (MS) detection of ricin in clinical samples has also significance[15-17]. Stability of ricin in beverages persists under certain conditions[18]. Solid-phase microextraction and headspace analysis and gas chromatography (GC/MS) also used to evaluate the method adopted for extraction of ricin[19]. In present work the possibility of finding ricin in medicinal castor oil samples studied by using the FTIR spectrometry analysis technique. Due to protein nature of ricin, specific peaks were appeared, that used for the characterization of ricin. While SDS-PAGE analysis used for confirmation of ricin. On quantification of ricin in medicinal castor oil an open threat of ricin poisoning evaluated. Unlike previously adopted method of agar dilution for antimicrobial activity of sulfonamides[20]. The minimum inhibition concentration of ricin was performed against the bacterial Strains *E. coli* and *Staphylococcus* for the evaluation of toxicity of purified ricin and medicinal castor oil samples.

## EXPERIMENTAL

The method of Despeyroux *et al.*[21] adopted for ricin extraction from castor seeds. Briefly, finally ground castor seed 100 g homogenized with 0.5 M NaCl 400 mL, pH maintained to 4 by $CH_3COOH$ and incubated for overnight at 25 °C. The pulp of castor seed was removed by centrifugation at 4000 rpm for 0.5 h. The supernatant was than treated with petroleum ether for removal of the lipid layer. Precipitation of ricin was carried out with 60 % $(NH_4)_2SO_4$ which then dialyzed against water. Ricin was further purified by gel filtration column chromatography by using superdex gel, 0.5 M NaCl and 0.5 M galactose. Obtained ricin was then oven dried below 40 °C and desiccated over $P_2O_5$. The ricin was obtained as white powder (RP1) and stored in vials. For crystallization of (RP1) the powder was redissolved and kept at ± 4 °C for 2 weeks. Crystals were then filtered, dried and preserved as (RC1).

A second method for extraction of ricin from castor seeds adapted was simple dilute acid method[22]. Briefly, 100 g of cleaned castor seeds were weighed and grinded carefully to avoid excessive heat. The obtained castor meal was treated with 500 mL of *n*-hexane to remove the oil contents. The oil free castor meal was then shade dried for 72 h. Castor meal then centrifuged with 200 mL of dilute $H_2SO_4$ (pH 3.8) at 3000 rpm for 0.5 h. The filtrate obtained and again to this castor meal 100 mL of dilute $H_2SO_4$ (pH 3.8) was added and kept overnight. Both filtrates were combined and pH of the filtrate was adjusted to 7.8 with the use of 12 % NaCl. Precipitation of protein was carried out with 20 % $Na_2SO_4$ and precipitates obtained through suction filtration. The precipitates then transfer to the dialysis tubing and dialysis was carried out for 24 h against distilled water to remove excessive salt (Distilled water changed twice). Both layers that present in the dialysis bag were collected *i. e*; water soluble and insoluble protein. Both fractions were then transferred to the crucibles and oven dried below 40 °C which further desiccated over $P_2O_5$. The ricin was obtained as white powder that slurred over $CCl_4$ and filtered. White ricin powder (RP2) was then dried and stored in vials. For crystallization of (RP2) the powder was redissolved and kept at ± 4 °C for 2 weeks. Crystals were then filtered, dried and preserved as (RC2).

**Extraction of ricin from medicinal castor oil samples:** Ricin is the side product in oil deriving procedure, so it is assumed safe for both medicinal and cosmetic uses[23]. Heat process for castor oil extraction is recommended due to possibility of inactivation of ricin while boiling[24], provided that sufficient extraction conditions applied with no cross contamination would occur. In decoction process of oil extraction, no precautional measures followed, because it is used for large scale production of castor oil (mainly for industrial uses), while cold pressed and filtered castor oil is free from ricin and recommended for the medicinal use.

Different medicinal brands of castor oil samples were collected from the pharmacies in locality of government and private hospitals of Lahore city. Total 16 medicinal castor oil samples were found to be used frequently. On the basis of ricin solubility, extraction of all medicinal castor oil samples (10 mL each) with 5 mL dilute $H_2SO_4$ (pH 3.8) were carried out by centrifugation at 4000 rpm for 0.5 h. All the obtained oil layers were scoped off and dilute $H_2SO_4$ (pH 3.8) layers (after treating with 2 mL petroleum ether) were kept in vials at ± 4 °C, for further analytical procedures.

**Gel electrophoresis (SDS-PAGE):** For the analysis of ricin on the basis of molecular weight gel electrophoresis was carried out. Trizma base ($C_4H_{11}NO_3$ f.w. 121.5) 4x running buffer of pH 6.8 and pH 8.8 were made for gel formations. 4.2 mL of acrylamide monomer, 100 µL of 10 % SDS, 100 µL of 10 % APS and 6 µL of TEMED were used in resolving gel formation. 0.65 mL of acrylamide monomer, 50 µL of 10 % SDS, 50 µL of 10 % APS and 3 µL of TEMED were used in the stacking gel formation. 2X-Loading dye for staining gels were made by 2 mL of 10 % SDS, 2 mL of running Buffer, 1.0 mL glycerol, 500 µL of BPB. β-Mercaptoethanol was used for the reduction of ricin. Reducing dye was prepared by adding 475 µL of 2x-loading Dye and 25 µL of β-mercaptoethanol. To the 75 µL of ricin samples 2x-loading Dye and β-mercaptoethanol was added and heat shocked. GE Healthcare ladder was used as biomarker and 100 µL of reducing dye was added to biomarker. 20 µL of reduced and non reduced samples of ricin and all the dilute acid extract medicinal castor oil samples were poured into the wells and run for electrophoresis at 100 V for 1 h. After completion of this process gels were stained for overnight and then transferred to destain solution (20 % methanol and 7 % acetic acid) to visualize the reduced and non-reduced ricin bands. Separation was done with Hoefer MS II OC1 electrophoresis apparatus.

**FTIR spectroscopy:** FTIR spectroscopic analysis was carried out by Thermo-Nicolet IR 200 spectrometer. Detection of ricin in all the dilute acid extracted medicinal castor oil samples performed over the KBr disks.

**UV-visible spectrophotometery:** The amount of ricin in dilute acid extracts of medicinal castor oil was measured by the photometric mode of Shimadzu 1700 UV-visible Spectrophotometer at $\lambda_{max}$ of ricin (279 nm) at 20 °C by using quartz cells. The system was calibrated with the bovine serum albumin (BSA) standard curve. The concentration of ricin present in samples was calculated by using the molar absorption coeffi-

cient of 93,900 L $mol^{-1}$ $cm^{-1}$. This molar absorptivity was calculated by contributing 69 % of tryptophan units and 31 % to tyrosine units of ricin structure[25].

**Minimum inhibitory concentration (MIC):** The agar medium was prepared as reported earlier[26]. 20 g of nutrient agar was dissolved in 1000 mL of distilled water and heated to mix well. Then agar medium was autoclaved at 121 °C for 15 min and poured into the plates. Then different volume (50, 100, 200, 500, 1000, and 2000 µL) of purified ricin and the dilute acid extracts of all 16 medicinal castor oil samples were introduced in agar plates and allowed to solidify for 20-25 min. Then these agar plates were streaked with loop full of *E. coli* (*Escherichia coli*) and *Staphylococcus aureus* bacterial strains and incubated at 37 °C for 24 h.

## RESULTS AND DISCUSSION

On precipitation of ricin protein by 20 % $Na_2SO_4$ and 60 % $(NH_4)_2$ $SO_4$, it was found that the ammonium sulphate precipitation give a higher amount of ricin than sodium sulphate precipitation, 3.8 g and 3.64 g, respectively.

Confirmation of extracted ricin from castor oil bean (RP1 and RP2) powdered ricin samples, (RC1 and RC2) crystalline ricin samples and medicinal castor oil dilute acid extract samples were done by SDS-PAGE. The reduced ricin samples (Fig. 1) appeared as two associated bands at 33 and 36 kDa of 60 % ricin A chain and 40 % ricin B chain respectively. While non reduced ricin (Fig. 2) appeared as a single band at about 66 kDa[27].

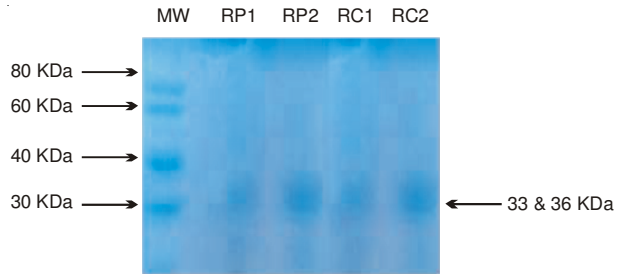


Fig. 1. Photograph of SDS-PAGE of the extracted ricin, with 2-mercaptoethanol. The first left column is ladder showing the molecur weight in kilodalton

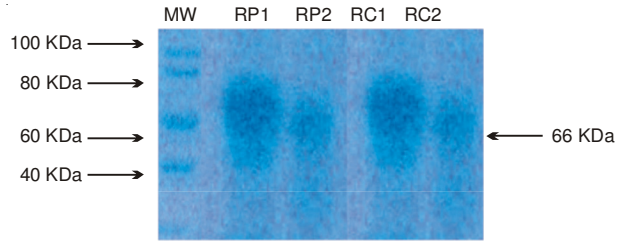


Fig. 2. Photograph of SDS-PAGE of non-reduced ricin, The first left column is ladder showing the molecur weight in kilodalton

**Characterization of ricin with FTIR spectroscopy:** FTIR spectrum of purified ricin and 16 medicinal castor oil extracts had found to show peaks in amino acid regions. Due to varying concentration of ricin, FTIR peaks of different intensities were obtained for all the samples a prominent peak revealed at about 1630 $cm^{-1}$ (Fig. 3) which represents the anti parallel β-sheet of protein oligomer of ricin[28] and it is also specific for recombinant ricin A chain[29]. Ricin gives smaller bands for other amino acids contents. C=S stretching peak appears at 1384 $cm^{-1}$, overtone and combination band for amino acid appears at 2076 $cm^{-1}$ and N-H vibration in resonance with amide II overtone band appears at 3300-3250 $cm^{-1}$.

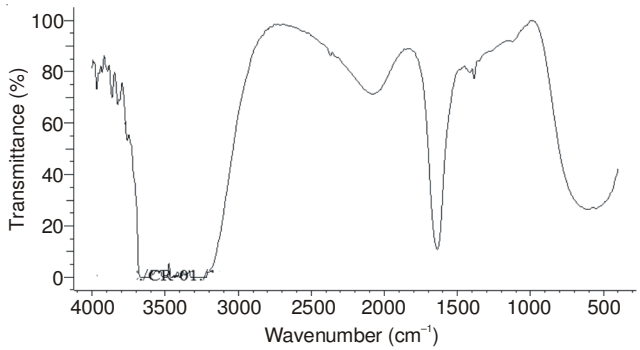


Fig. 3. FTIR spectra for ricin

**Determination of amount of ricin:** A known ricin concentration of 0.08 mol $L^{-1}$ (4.8 mg $L^{-1}$) and unknown concentrations of ricin present in the dilute acid extracts of all the medicinal 16 castor oil samples were calculated by measuring absorption at 279 nm on UV-visible spectrophotometer at ± 20 °C. The recorded absorbance was then correlated with the absorbance of known concentration of ricin. The amount of ricin found (Table-1) in castor oil samples was determined, by using the molar absorption coefficient 93,900 L $mol^{-1}$ $cm^{-1}$ [28] and it was found between the 0.671 mol $L^{-1}$ to 0.713 mol $L^{-1}$. Further a graph (Fig. 4) for the varying amount of ricin in different marketed castor oil samples was plotted.

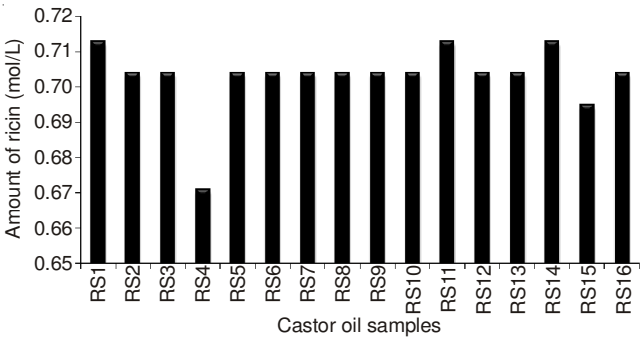


Fig. 4. Ratio of ricin in different castor oil sample in mol/L

**Determination of MIC value for ricin against bacterial growth:** The toxicological effect of ricin was observed on the microbial growth, where ricin shows inhibition effects against the *E. coli* strain. Purified ricin and all the 16 medicinal castor oil showed antimicrobial activity (Table-2) against bacterial Strain *E. coli* at 500 µL and no antimicrobial activity for *Staphylococcus* upto 2000 µL.

The extraction of ricin was carried out by salt extraction method followed by gel filtration and simple dilute acid method followed by precipitation and re-crystallization for purification of ricin. Both the adopted methods were found effective for obtaining the purified ricin on protein estimation by the lowry method. SDS-PAGE confirmed the reduced and non-reduced ricin by their respective molecular weights.

TABLE-1
CALCULATED AMOUNT OF RICIN IN DIFFERENT MEDICINAL CASTOR OIL SAMPLES

| S. No. | Castor oil brands | Sample codes | Concentration (mol/L) | Concentration (mg/L) | Concentration (mg/10 mL) |
|---|---|---|---|---|---|
| 1 | Ovals Pharmaceuticals | $RS_1$ | 0.713 | 42.76 | 0.43 |
| 2 | Micko's Industries | $RS_2$ | 0.704 | 41.46 | 0.41 |
| 3 | Shamsi Pharmacy | $RS_3$ | 0.704 | 41.46 | 0.41 |
| 4 | Zain Laboratories Limited | $RS_4$ | 0.671 | 40.24 | 0.40 |
| 5 | Life styles Pharmaceutics | $RS_5$ | 0.704 | 41.46 | 0.41 |
| 6 | Merhaba Industries | $RS_6$ | 0.704 | 41.46 | 0.41 |
| 7 | Mehmood Pharmacy | $RS_7$ | 0.704 | 41.46 | 0.41 |
| 8 | Anmol Pharmacy | $RS_8$ | 0.704 | 41.46 | 0.41 |
| 9 | Mehmood Ali Pharmaceutics | $RS_9$ | 0.704 | 41.46 | 0.41 |
| 10 | Subhan Herbal Pharmacy | $RS_{10}$ | 0.704 | 41.46 | 0.41 |
| 11 | Husnain Pharmaceutics | $RS_{11}$ | 0.713 | 42.76 | 0.43 |
| 12 | Lahore Pharmacy | $RS_{12}$ | 0.704 | 41.46 | 0.41 |
| 13 | Airop-Lane Brand | $RS_{13}$ | 0.704 | 41.46 | 0.41 |
| 14 | Mubarac Pharmacy | $RS_{14}$ | 0.713 | 42.76 | 0.43 |
| 15 | Qarshi Industries | $RS_{15}$ | 0.695 | 41.68 | 0.42 |
| 16 | Darul Sehat Herbal Pharma | $RS_{16}$ | 0.704 | 41.46 | 0.41 |

TABLE-2
ANTIMICROBIAL ACTIVITY OF PURIFIED RICIN AND CASTOR OIL EXTRACTED SAMPLES $RS_1$ TO $RS_{16}$

| S. No. | Volume of ricin extract (μL) | Antimicrobial activity against | |
|---|---|---|---|
| | | *Escherichia Coli* | *Staphylococcus* |
| i | 50 | Growth | Growth |
| ii | 100 | Growth | Growth |
| iii | 200 | Growth | Growth |
| iv | 500 | No growth | Growth |
| v | 1000 | No growth | Growth |
| vi | 2000 | No growth | Growth |

Generally secondary structure of protein had been identified by transmission FTIR in the region 1700-1500 $cm^{-1}$ [30]. It was found that proteins including ricin have specific peaks which are due to the contribution of difference in amino acid contents and different side chains[31]. IR spectrum in the range of 1700-1600 $cm^{-1}$ is attributed to the amide I bond[32] and secondary structure of protein ranges from 1640-1625 $cm^{-1}$ for β sheet and 1628-1610 $cm^{-1}$ for α-helices[33]. While on analysis the stability of the recombinant ricin A chain (RTA) by FTIR spectroscopy gives a prominent peak[29] at 1630 $cm^{-1}$. In ricin FTIR spectrum only few other transmission bands were found.

Calculation of the amount of ricin was carried out by UV visible spectroscopy and highest amount of ricin was found in the three medicinal castor oil samples RS1, RS11 and RS14. Slightly lower and approximately same amount of ricin contamination was found in eleven samples (RS2, RS3, RS5, RS6, RS7, RS8, RS9, RS10, RS12, RS13, RS16). A decrease in ricin amount in sample RS15 was observed and least amount of ricin was found in RS4.

Sulfonamide a renowned antimicrobial agent showed good activity against gram positive bacteria[20] while ricin a toxin showed antimicrobial activity against gram negative bacteria. This difference in antimicrobial activity is accounted for difference in cell wall composition of both bactreial strains. The *E. coli* is gram negative bacteria specie with the lipopolysaccharides on the cell wall that allows ricin B chain to attach on the outer cell wall and introduce the ricin A chain into the cytosol to inactivate ribosome. *Staphylococcus* is gram positive bacteria specie and had thick peptidoglycan layer, probably this is the reason for no observed antimicrobial effect of ricin on *staphylococcus* strain.

## Conclusion

Ricin is most toxic on inhalation and parentarel injection, then ingestion. Skin and eye exposure with castor beans dust or pomace results in allergic reactions[34]. While on ingestion ricin is not much toxic probably because of its poor intestinal absorption. Oral intake of ricin has not been much studied. On oral ingestion of ricin all the gastro-intestinal symptoms has been found and on experimentation for determining the lethal oral dose (1-20 mg/kg of body weight) it has been concluded that ricin, within few hours of intake, founds its way into the blood to the body tissues[35].

In the present experimental work the maximum amount of ricin present in medicinal castor oil samples was 0.43 mg/10 mL and minimum amount was found 0.40 mg/10 mL (10 mL equals to 1 table spoon). Although it is found less than the oral lethal dose of ricin intake in human but this does not eliminate the threat of ricin exposure, as it largely depend on age and amount of ricin intake, so ricin cross contamination in castor oil make it unsafe for medicinal and cosmetic use.

Enzymatic mode of action and extra stability of ricin also enhances the level of intoxication of such medicinal castor oil's. During this research work an attempt was made to study the MIC of the ricin in two different types of bacterial strains. The bacterial growth inhibition effect of ricin is directly correlated to its structural specification (enzymatic mode), as free glycopeptides assist the attachment of ricin to the cell surface which largely present in gram negative bacteria.

According to Food and Drug Administration (USA) castor oil is over the counter laxative remedy. The castor oil is widely used as skin conditioning product in cosmetics, also a surfactant tool. On general hospital survey, observed side effects of castor oil ingestion are abdominal cramps and pain, diarrhea, nausea, vomiting. Long term use can result in loss of fluid and electrolytes leading to death. The safety of castor oil use remains a big question in Europe and America and only united state pharmacopeia (USP) standard castor oil is recommended for use in cosmetic industry and in medicine as

a laxative. Despite of this, still in Asia no prevention measures taken and even some herbal medicine that contains the castor meal and are being used.

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