DEVELOPMENT OF NON-TOXIC LATEX FORMULATION FOR BIOMEDICAL APPLICATIONS

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Objectives

a) Evaluation of commercially available NRL products.
b) Screening of latex additives individually for its toxicity potential.
c) Selection of appropriate additives based on the toxicity data.
d) Preparation of vulcanised sheets of known composition using the additives selected.
e) Evaluation of mechanical properties of the above sheets and selection of appropriate composition.
f) Study of the migrating behaviour as well as toxicity (by *in vitro* and *in vivo* methods) of latex formulation selected.
g) Determination of dermal allergenicity (Sensitisation test) of the selected formulation in guinea pigs.
h) Evaluation of tissue compatibility (Intramuscular implantation test) of the selected formulations in rabbits.

Experimental

Commercial brands of surgical/examination gloves (pre-powdered and powder free) made of natural rubber latex were collected from local sources. Double centrifuged natural rubber latex (60% dry rubber content) was obtained from local suppliers. Rubber chemicals such as zinc diethyldithiocarbamate (ZDEC) and zinc dibutyldithiocarbamate (ZDBC) were supplied by National Organic Chemical Industries Limited (NOCIL, Mumbai, India). Zinc dibenzyldithiocarbamate (ZBEC), zinc mercaptobenzothiazole (ZMBT) and 4-methyl 2-mercaptobenzimidazole (MMBI) were obtained from Bayer AG, Germany. Wingstay L (reaction product of butylated p-cresol and cyclopentadiene) was purchased from Plasti Chem Pvt India, India. Chemicals such as dichloromethane, acetone, hexane, ether, sodium chloride and urea were procured from local sources. Lactic acid was procured from Sigma Aldrich, U.S.A.

Evaluation of Commercial Gloves

In vitro cell culture cytotoxicity test

The surgical/examination gloves (pre-powdered and powder free) were code named with English alphabets. Cytotoxicity potential of these gloves was evaluated by both direct contact test and test on extract assay as per ISO 10993-5 (1992). The procedure for the cytotoxicity test is detailed later in this report.

Acetone extraction

The gloves were cleaned to remove the powder and other dust particles and subjected to soxhlet extraction according ASTM D 297-93 using acetone as the extraction medium. About 2 g of the samples are cut in small pieces and extracted with acetone using a soxhlet extractor for 16 h. The

residue is recovered by evaporating the acetone on a water bath at 60 °C and the percentage residue is calculated.

FT-Infrared Spectroscopy

The FT-IR spectrum of the acetone extract of brand 'A' was taken using Nicolet Impact 410 Spectrophotometer using KBr pellet method.

Extraction of gloves in artificial sweat

A quantitative estimation of dithiocarbamate released in artificial sweat from latex gloves was carried out. Artificial sweat was prepared according to the composition given in European standard EN 1811:1998(E) (Table 1).

Table 1: Composition of artificial sweat

Ingredient	Concentration (wt %)
Sodium chloride	0.5
Lactic acid	0.1
Urea	0.1
Deionized water	99.3

The pH of the sweat solution was adjusted to 6.5 ± 0.1 by adding a 1% solution of ammonia. The extraction was carried out at 37 ± 2 °C for 24 h with constant mechanical shaking. The sweat to rubber ratio (in g) was maintained at 50. The resultant solution was extracted with dichloromethane. The dichloromethane was distilled off to recover the residue.

Estimation of sweat-extractable dithiocarbamates in gloves

The residue obtained after sweat extraction was re-dissolved in a known quantity of dichloromethane and reacted with an aqueous ammoniacal solution of cupric sulphate. The dichloromethane layer was separated out and distilled off. The solid copper- dithiocarbamate complex obtained was re-dissolved in acetone and subjected to HPLC analysis to estimate the quantity of dithiocarbamate released in artificial sweat.

Before running HPLC, the absorption wavelength of the copper-dithiocarbamate complex was determined by running an UV spectrophotometer (Shimadzu, UV-1601). The complex was found to be absorbing at 435nm. Using 435nm as detection wavelength, the estimation was carried out using a Waters HPLC system equipped with a 510 pump, C18 column and 7725 Rheodyne injector. The mobile phase used was acetone-water in the ratio 90:10 with a flow rate of 1 ml/min. Known quantities of ZDEC and ZDBC was taken and converted into the copper complex which served as standards. The identification and estimation of amount of dithiocarbamate migrated into artificial sweat was estimated by comparison with the standard.

Estimation of water extractable proteins in gloves

The water extractable protein content of some brands of latex gloves was determined according to ASTM D 5712-99. The various steps involved were the standard preparation using chicken egg albumin, extraction of gloves in phosphate buffer, precipitation of proteins with alkali and acid and color development. Also, a background correction for the chemicals, which may interfere in Lowry protein assay, was also conducted for the gloves.

A series of standard solutions of chicken egg albumin (Grade V, Sigma) of concentrations from 2 to 200 µg/ml was prepared. The gloves were extracted in phosphate buffer at pH 7.4 ± 0.2 for 120 ± 5 min. The proteins present in the different standard solutions and test samples were precipitated with sodium deoxycholate and phosphotungstic acid/trichloroacetic acid mixture. The precipitated proteins were then redissolved in a specified amount of sodium hydroxide. It was then followed by the addition of alkaline sodium tartarate and folin reagent for color development. The absorbance of the solutions was read at 750 nm. The unknown protein contents were calculated from the standard protein curve fitted to second degree polynomial equation.

Intracutaneous Irritation Potential of Dithiocarbamates

This study was aimed to determine the intracutaneous irritation potential of three commonly used dithiocarbamates *viz.* zinc diethyldithiocarbamate (ZDEC), zinc dibutyl dithiocarbamate (ZDBC) and zinc dibenzyldithiocarbamate (ZBEC). The test was performed as per ISO 10993-10 : 1995 (E). Solutions of the three accelerators were prepared to get concentrations of 320, 560 and 960 μg/ml. A 0.2 ml of the above solution was injected into rabbits and a detailed procedure is explained later in this report. The irritation potential (erythema and edema) was scored at the end of 24, 48, 72 hrs and the average skin irritation score was calculated.

Natural Rubber Latex Formulations

Natural rubber latex compositions were prepared using different dithiocarbamate accelerators. Chemicals such as sulphur, zinc oxide, ZDEC, ZDBC and 4-methyl 2-mercaptobenzimidazole (MMBI) were added into the latex as 50% dispersions in water. Potassium hydroxide was added as 10% aqueous solution. The additives were added into the latex on measures of 'number of parts of additive per 100 part of rubber (p.p.h.r)' on dry weight basis. All the dispersions were added to the latex one by one in the same order as given in the formulation recipes with 10 min interval between each addition. The compounded latex was matured for 19 h, and cast on leveled glass plates. The cast sheets were allowed to air dry for at least 48 h. The dried sheets were then vulcanized at 120 °C for 15 min followed by post-leaching in hot water at 50 °C for 15 min for four times. The water was changed at every 15 min interval during the leaching process. In addition, an additional leaching in water was given for 24 h. The sheets were dried in an air oven at 70 °C for 30 min.

Ingredients*	Formulation code						
	DCE-1	DCE-2	DCE-3	DCE-8	DCE-10	DCE-12	DCE-12AO
60% NRL	100	100	100	100	100	100	100
10% KOH	0.3	0.3	0.3	0.3	0.3	0.3	0.3
50% Sulphur	0.5	0.5	0.5	0.5	1.0	0.75	0.75
50% ZDEC	0.5	0.66	0.82	1.0	0.5	0.5	0.5
50% ZnO	0.5	0.5	0.5	0.5	0.5	0.5	0.5
50% MMBI	-	-	-	-	-	-	0.8

The formulations containing ZDEC are given in Table 2. Here, the amount of ZDEC was varied from 0.5-1.0 p.p.h.r keeping the amount of sulphur and zinc oxide constant at 0.5 p.p.h.r (formulations DCE-1, 2, 3 & 8). In the next step, the amount of ZDEC was kept at a lower level of 0.5 p.p.h.r and the amount of sulphur was varied (formulations DCE-1, 10 & 12). An antioxidant (MMBI) was added in one of the latex compositions (DCE-12AO).

Table 2: Formulations using ZDEC

** On dry weight basis*

(Note: DCE-12 and DCE-12AO were coded as E-12 and E-12AO respectively in biological tests)

Table 3 gives the various formulations employing ZDBC. As in the previous case, the ZDBC amount was varied from 0.19-0.82 p.p.h.r keeping the sulphur content constant at 0.5 p.p.h.r (formulations BU-1, 2, 3 & 4). In the next step, the ZDBC content was kept constant at 0.5 p.p.h.r and varied the amount of sulphur (formulations BU-1, 5, 6 & 7).

Table 3: Formulations using ZDBC

Ingredients*	Formulation code						
	BU-4	BU-1	BU-2	BU-3	BU-5	BU-6	BU-7
60% NRL	100	100	100	100	100	100	100
10% KOH	0.3	0.3	0.3	0.3	0.3	0.3	0.3
50% Sulphur	0.5	0.5	0.5	0.5	0.75	1.0	1.5
50% ZDBC	0.19	0.5	0.66	0.82	0.5	0.5	0.5

50% ZnO	0.5	0.5	0.5	0.5	0.5	0.5	0.5

** On dry weight basis*

Table 4 gives the formulations employing ZBEC as the vulcanization accelerator. As explained in the above cases, the effect of varying amounts of ZBEC at constant sulphur content has been studied (BZ-1, 2, 3 & 4). Since the use of ZBEC alone does not give appreciable enhancement in the tensile properties, a secondary accelerator, namely ZMBT, has been employed. The formulations (BZ-5, 6 & 8) employed fixed level of ZMBT (0.2 p.p.h.r) and ZBEC (0.5 p.p.h.r) for varying amount of sulphur.

Table 4: Formulations using ZBEC

Ingredients*	Formulation code						
	BZ-4	BZ-1	BZ-2	BZ-3	BZ-5	BZ-6	BZ-8
60% NRL	100	100	100	100	100	100	100
10% KOH	0.3	0.3	0.3	0.3	0.3	0.3	0.3
50% Sulphur	0.5	0.5	0.5	0.5	0.5	0.75	1.0
50% ZBEC	0.19	0.5	0.66	0.82	0.5	0.5	0.5
50% ZMBT	-	-	-	-	0.2	0.2	0.2
50% ZnO	0.5	0.5	0.5	0.5	0.5	0.5	0.5

** On dry weight basis*

Characterization of Latex Sheets

The vulcanized latex sheets were characterized for their tensile properties (tensile strength, elongation at break, modulus at 100 and 300% elongation), crosslink density and amount of ZDEC released into dichloromethane and artificial sweat.

Evaluation of tensile properties

Dumbbell shaped specimens were punched from the vulcanized sheets using pneumatic die cutter (ATSFAAR, Italy) with an ISO Type 2 die. The testing was carried out in an Instron Universal Testing Machine, Model 4411, at a strain rate of 10 min^{-1}. Accelerated ageing was carried out on selected formulations to study the extent of retention of mechanical properties. For this, the samples were aged at a temperature of 70 ± 2 $^{\circ}C$ for a period of 168 h in an air oven and evaluated the tensile properties as mentioned above.

Estimation of DCM-extractable ZDEC

Among the different latex formulations, latex sheets containing ZDEC was subjected to extraction in dichloromethane to determine the extent of release of ZDEC. The vulcanized latex sheets were extracted in dichloromethane (DCM) for 24 h at room temperature. The extract was evaporated and a residue was obtained. ZDEC present in the residue was quantitatively analyzed using HPLC as its copper complex. The procedure for this is already detailed in the previous section.

Estimation of sweat-extractable ZDEC

A quantitative estimation of ZDEC released into artificial sweat from latex vulcanizates was carried out. The estimation was carried out after converting ZDEC into its respective copper complex and procedure for the determination of sweat-extractable ZDEC is already explained in the previous section.

Biological Characterization

In vitro cell culture cytotoxicity test

For the *in vitro* cell culture cytotoxicity test L929 mouse fibroblast cell lines procured from National Centre for Cell Science (NCCS), Pune, India was used. All the test samples were sterilized by ethylene oxide gas before cell culture cytotoxicity test. The latex sheet was subjected to both direct contact and test-on-extract assays to evaluate their toxicity towards mouse fibroblast L929 cells. Both the tests were performed according to ISO 10993-5:1999 (E) standard.

The L929 cell lines were maintained in RPMI-1640 culture medium (Hi Media, Pune, India) supplemented with 10% fetal bovine serum (Sigma, U. S. A), 100 IU/ml penicillin and 100 µg/ml medical grade streptomycin. The required number of cells was sub-cultured in a multi-well plate and incubated at 37 ± 2^0C in 95% humid and 5% carbon dioxide atmosphere with a medium change at 3 days interval.

In the direct contact assay, the glove material was placed in contact with the cells and incubated at 37 ± 2ºC for 24 h. Here the test sample was placed at the centre of the well so that one-tenth of the cell layer surface was covered. Copper and tissue culture grade polystyrene (TCPS) were used as the positive and negative controls respectively. The degree of cytotoxicity was determined qualitatively by viewing general morphology, vacuolization, detachment of cells and cell lyses around the material under an inverted phase contrast microscope (Leica, DMIL). Based on these parameters, the material was graded according to a cytotoxicity scale ranging from 0-3; '0' for non-cytotoxic (negative control), '1' for mildly cytotoxic, '2' for moderately cytotoxic and '3' for severely cytotoxic (positive control).

The test on extract assay assesses the toxicity of the glove extract. The glove extract was prepared at 37 ± 2°C by incubating 0.1 g of the glove in 1 ml of the culture medium supplemented with fetal bovine serum. The duration of extraction was 5 days. A monolayer of cells grown in a multi-well plate containing the cell culture medium was taken and the medium was drained off completely. The glove extract was added carefully to the multi-well plate without disturbing the cell monolayer. This was then incubated at 37 ± 2°C for 24 h. The cell response was compared and graded in the cytotoxicity scale as described above. Diluted phenol and TCPS were used as the positive and negative controls respectively.

Hemolysis

The hemolytic properties of latex material were assessed as per ASTM F 756-93 using rabbit blood. Material specimens and their extracts are exposed to contact with rabbit blood under defined static and dynamic conditions and the increase in plasma hemoglobin is measured. Anticoagulated (using ACD as anticoagulant) rabbit blood was collected from three donors and diluted with normal saline to adjust the hemoglobin content to less than 25 ± 2.5 mg/ml. The test specimens were cut into 50 rectangular strips of 10x1 mm^2 size to give an equivalent surface area of 15.7 cm^2. The material extract (15.7 cm^2/5 ml saline) was prepared in normal saline at 70 °C for 24 h. Both the material and material extract was subjected to static and dynamic test. For static test, 4 ml of the material extract was added into a 5ml blood substrate and incubated for 4 h at 37 °C. In the case of direct test, a 15.7cm^2 of the material was placed in direct contact with 5 ml of blood substrate and incubated for 4 h at 37 °C. The blood samples were then centrifuged at 100-200 ×G for 15 min followed by recentrifugation at 700-800 ×G for 5 min. The supernatant liquid was analyzed for hemoglobin content using a spectrophotometer at 540 nm. The hemolytic index was calculated using the formula,

$$H.I = \frac{Hemoglobin released\,(mg/ml)}{Hemoglobin present\,(mg/ml)} \times 100$$

For dynamic test, the material and the material extract was incubated with blood substrate at 37 °C for 1 h with constant shaking at 30 ± 6 r.p.m. The results were compared with that of a negative control, which was low density polyethylene. For the test to be valid, the negative control must have a hemolytic index of less than 1.2%. The test material meets the requirement of the test if the hemolytic index is found to be less than 2%.

Intracutaneous (Intradermal) Reactivity test

Intracutaneous irritation studies were carried out to evaluate the local responses to the extracts of the vulcanised latex sheets following intracutaneous injection into rabbit. Healthy, thin skinned New Zealand albino rabbits of either sex, with body weight not less than 2 kg, were used for the study. The animals for the study were prepared by clipping the fur on the dorsal side, close to the skin. Extracts from the material were prepared both in normal saline and cottonseed oil as per ISO 10993-10:1995 (E). For this, a 60 cm^2 of the sterilized sample was extracted in 10 ml each of

physiological saline and cotton seed oil at 70°C for a period of 24 h. Three Rabbits are used for the test. A 0.2 ml of physiological saline extract of the latex sheets was injected at five anterior sites on the left side of each rabbit (Figure 1).

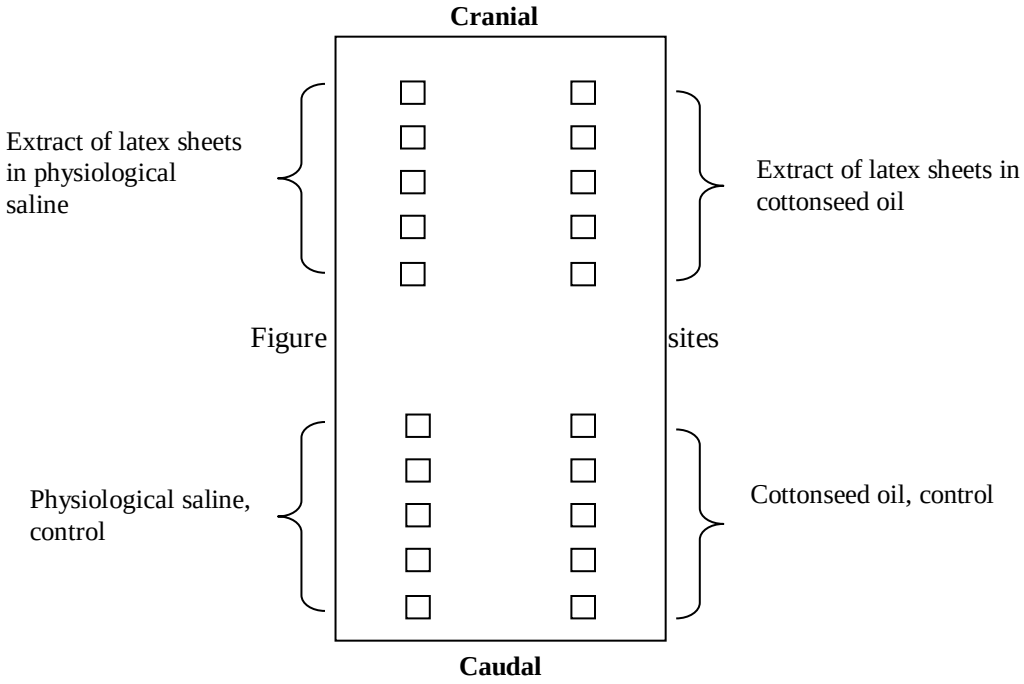


Figure sites

Similarly, 0.2 ml of physiological saline control was injected at five posterior sites on the same side of each rabbit. The above procedure was repeated for cotton-seed oil (CSO) extracts of the latex sheets and control solutions on the right side of each rabbit. The sites were then evaluated for skin reactions at 24, 48, and 72 hours following the injection. Toxicity associated symptoms such as erythema, and oedema 24, 48, and 72 hours were recorded and average irritation score was calculated. The primary irritation index was also calculated. The primary irritation index is categorized into four as given in Table 5 a .

Table 5a: Primary irritation responses categories in rabbit

Grade	Index*
Negligible	0 to 0.4
Slight	0.5 to 1.9
Moderate	2 to 4.9
Severe	5 to 8

** The index is determined by adding the primary irritation score of each site of the animal and dividing the total score by the number of animals and number of periods*

The **i**ntracutaneous irritation experiment was repeated using artificial sweat, polyethylene glycol 400, alcohol/saline (1:20) as the extraction medium according to ISO 10993-10: 2002 (E). The test is same as ISO 10993-10:1995(E) except it is conducted in two rabbits. Grading of tissue reaction for erythema and oedema was done at each observation period as per the classification system given in Table 5b for each injection site and at each time interval observed, and the results were recorded. After 72h grading, all erythema grades plus oedema grades are totaled separately for each test sample and control. Each of the totals is divided by 12 (2 animals × 3 grading periods × 2 grading categories). The overall mean score for each test sample versus each corresponding control is determined.

Table 5b: Grading system for Intracutaneous (intradermal) reactions as per ISO 10993-10:2002(E).

Erythema	**Score**	**Oedema**	**Score**

No erythema	0	No oedema	0
Very slight Erythema (barely perceptible)	1	Very slight oedema (barely perceptible)	1
Well-defined erythema	2	Well –defined oedema (edges of area well-defined by definite raising)	2
Moderate erythema	3	Moderate oedema (raised approximately 1mm)	3
Severe Erythema (beet –redness to eschar formation preventing grading of erythema)	4	Severe oedema (raised more than 1mm and extending beyond exposure area)	4
Total possible score for irritation			**8**
Note : Other adverse changes at the injection sites shall be recorded and reported			

The requirements of the tests are met if the difference between the test sample mean score and the control mean score is 1.0 or less. The test would have been repeated, if at any observation period the average reaction to the test sample is questionably greater than the average reaction to the control

Sensitization studies

Skin sensitization by closed patch sensitization method was carried out in guinea pigs to evaluate the potential of the latex vulcanizate in inducing sensitization. The material extract was prepared by extracting a 60 cm^2 of the sterilized sample in 10 ml each of physiological saline at 70°C for a period of 24 h**.** Four-ply gauze was used as the control material. A total of 15 Hartley strain guinea pigs, weighing between 300-500 g of either sex were selected for the experiment. Prior to each application period, the fur on the dorsal area on either side of the vertebral column of each animal was clipped. The test was carried out in two phases-an induction phase followed by a challenge phase. In the induction phase, the skin was lightly swabbed with 70% alcohol and air dried. A saturated patch of four ply gauze in physiological saline extract of the material was applied topically on the clipped upper back region of 10 animals (test animals). Similarly a saturated patch of four ply gauze in physiological saline was topically applied as control to other 5 animals. This was then covered with occlusive dressings. The occlusive dressings from the test and the control were removed after 6 h. The reaction at the site of application was observed at 24 h and 48 h for the evidence of any erythema and oedema. This procedure was repeated at weekly intervals for three more weeks.

Fourteen days after the last application (induction period) all the test and control animals are challenged with the physiological saline extract of the test material. For this, the hair on the animal's flank area (untested area) was removed and lightly swabbed with 70% alcohol and topically applied a saturated patch of test material. This was then covered with occlusive dressings. The occlusive dressings from the test and the control were removed after 6 h. The reaction at the site of application was observed at 24 and 48 h for the evidence of any erythema and oedema. The numerical grading was recorded as per ISO 10993-10: 1995(E).

Results

Evaluation of Commercial Gloves

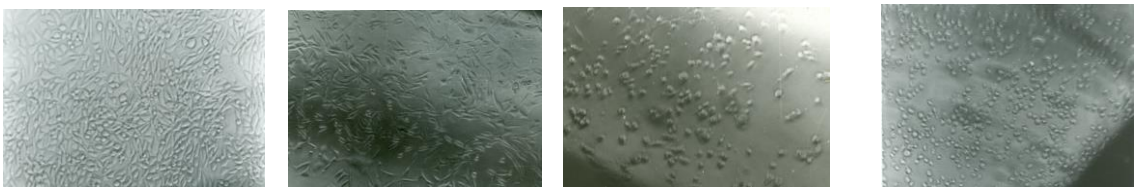
Of the eleven brands of gloves tested, eight were of surgical type and three were of examination type glove. The cytotoxicity scores and the percentage acetone residue of the different brands of natural rubber gloves are given in Table 6.

Table 6: Cytotoxicity scores and percentage acetone residue of different brands of latex gloves'

Sl.No.	Brand code	Glove type	Direct contact	Test on extract	Acetone extract (%)
1	A	Surgical, pre powdered	1	0	5.0
2	B	Surgical, powder free	3	0	3.7
3	C	Surgical, pre powdered	1	1	3.5
4	D	Surgical, pre powdered	2	*a*	3.8
5	E	Surgical, pre powdered	0	0	3.8
6	F	Surgical, pre powdered	0	0	3.1
7	G	Surgical, pre powdered	0	3	3.5
8	H	Surgical, pre powdered	3	3	-
9	I	Examination, powder free	0	0	3
10	J	Examination, powder free	0	0	3.3
11	K	Examination, powdered	3	3	3.3

0-non-cytotoxic; 1-mildly cytotoxic; 2-moderately cytotoxic; 3-severely cytotoxic
a-tests that are not conducted

The cytotoxicity assay showed that nearly 64% of the total gloves selected were cytotoxic to mammalian fibroblast cells. Of these, 75% were surgical and 33% were examination gloves. Typical cell responses to the gloves are shown in Figure 2.

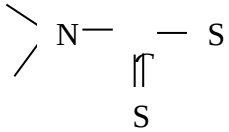
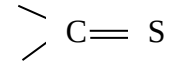


A B C D

Figure 2. Cytotoxic responses of latex gloves'. A: Non cytotoxic; B: Mildly cytotoxic; C: Moderately cytotoxic; D: Severely cytotoxic

The cell response was determined from the morphological change and cytopathic or cell death nature exhibited by the cell line. The intact cells were distinguished by their spindle shape whilst the damaged cells appeared in spherical shape. The cytopathic effect of the glove samples was indicated by the presence of remnants of the dead cells.
The FT-IR spectroscopic analysis of the acetone extract of brand 'A' showed the presence of zinc diethyldithiocarbamate (ZDEC) after comparing with a control spectrum of ZDEC. Table 7 gives the IR absorbance peaks of brand 'A', Control ZDEC and the standard peaks for the dithiocarbamate and C=S group.

Table 7: FT-IR absorbance peaks of brand 'A' and control ZDEC

	Standard peak (cm^{-1})	Brand 'A' (cm^{-1})	ZDEC (cm^{-1})
	1050	1073	1073
	1200-1050	1121	1119

The chromatogram of the sweat extract of gloves showed a peak with a retention time corresponding to that of ZDEC indicating that ZDEC residues were released into artificial sweat from the gloves. Figures 3(a) and 3(b) give the relationship between the sweat-extractable ZDEC and degree of cytotoxicity of latex gloves in the direct contact assay and test on extract assay respectively.

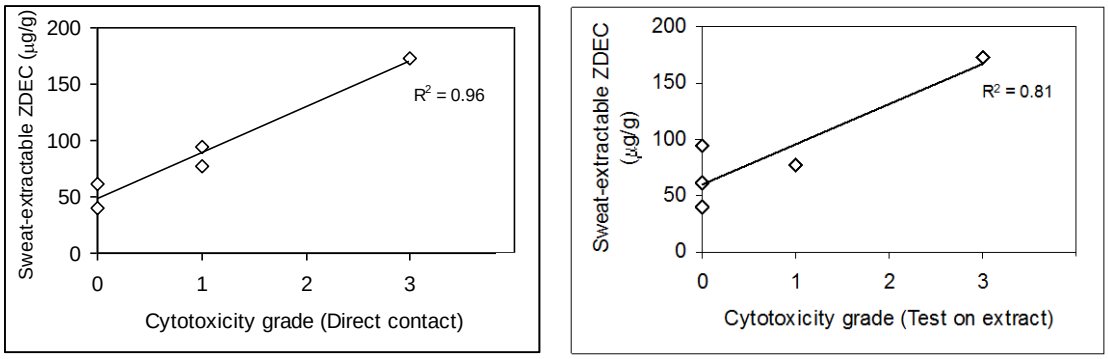


Figure 3. Plot of cytotoxicity grade Vs. sweat-extractable ZDEC. (a) Direct contact assay, (b) Test on extract

Figures 3(a) and 3(b) showed a linear relationship existing between the sweat-extractable ZDEC and the two types of *in vitro* cell culture methods indicating that the cytotoxic potential of the gloves increased with an increase in the amount of sweat-extractable ZDEC. Figure 3(a) showed that a non-cytotoxic response was evident in gloves having extractable dithiocarbamates equal to or less than 61 µg/g of glove. A mildly cytotoxic behavior was observed when the amount of sweat-extractable ZDEC was in the range 78-94 µg/g of glove. The material was severely cytotoxic when the sweat-extractable ZDEC rose to about 173 µg/g of glove. The values of the correlation coefficients between the sweat-extractable ZDEC and cytotoxicity grade were 0.96 and 0.81 for direct contact assay and test on extract assay respectively. This follows that the amount of leachable and toxic ZDEC significantly influences cytotoxic potential of the latex gloves.

The amount of water extractable proteins present in some of the commercial brands of gloves is shown in Table 8.

Table 8: Protein content of some brands of gloves'

Brand code	Extractable proteins	
	µg/dm^2 of glove	µg/g of glove
A	260 ± 40	243 ± 23
C	158.6 ± 1.6	151 ± 1.2
D	143.2 ± 44	101.4 ± 30.6
E	136.1 ± 1.8	158.95 ± 2
I	22.7 ± 6.89	25.5 ± 6.9

The amount of total extractable proteins in the gloves ranged from 25.5 to 243 µg/g of glove.

Intracutaneous Irritation Potential of Dithiocarbamates

It was found that the order of skin irritation potential was in the order ZDEC>ZDBC>ZBEC. This result could be used for the selection of additives during latex compounding stage so that the final product may produce minimum irritation.

Natural Rubber Latex Formulations

Figure 4 shows the effect of varying level of ZDEC on tensile properties of latex vulcanizates.

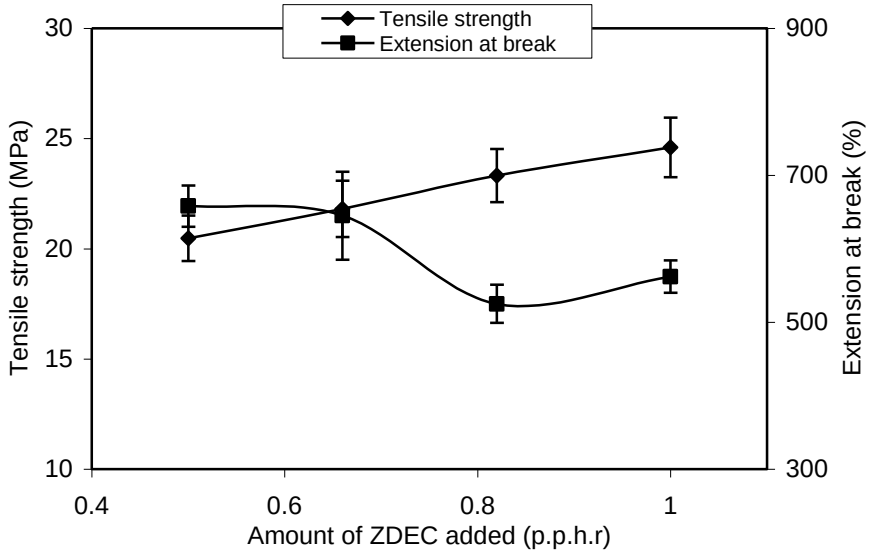


Figure 4: Effect of varying amount of ZDEC on tensile strength and extension at break

Figure 5 shows the influence of varying amount of sulphur on the mechanical properties of the latex sheets prepared from various formulations as given in Table 2.

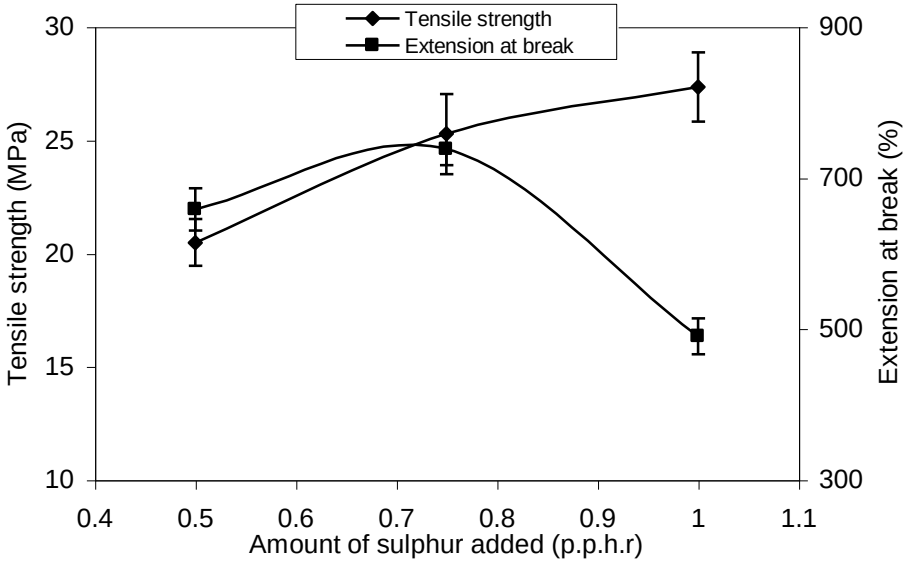


Figure 5: Effect of varying amount of sulfur on tensile strength and extension at break

It was apparent that the tensile strength tends to increase with an increase in either ZDEC or sulphur content. However, the magnitude of increase was high when the sulphur content was increased while maintaining the level of ZDEC a constant (Figure 5). The modulus at 100 and 300% extension of all the latex vulcanizates was less than 5.5 MPa, the maximum limit put forward by ASTM D 3577-00. Among all the formulations studied, only one formulation (DCE-12) met the minimum requirements put forward by the ASTM for surgical gloves.

Figure 6 shows the effect of varying amount of ZDBC on the tensile strength and extension at break of the latex sheets when sulphur was kept constant at 0.5 p.p.h.r. The effect of varying amount of sulfur on the tensile strength and extension at break was given in figure 7.

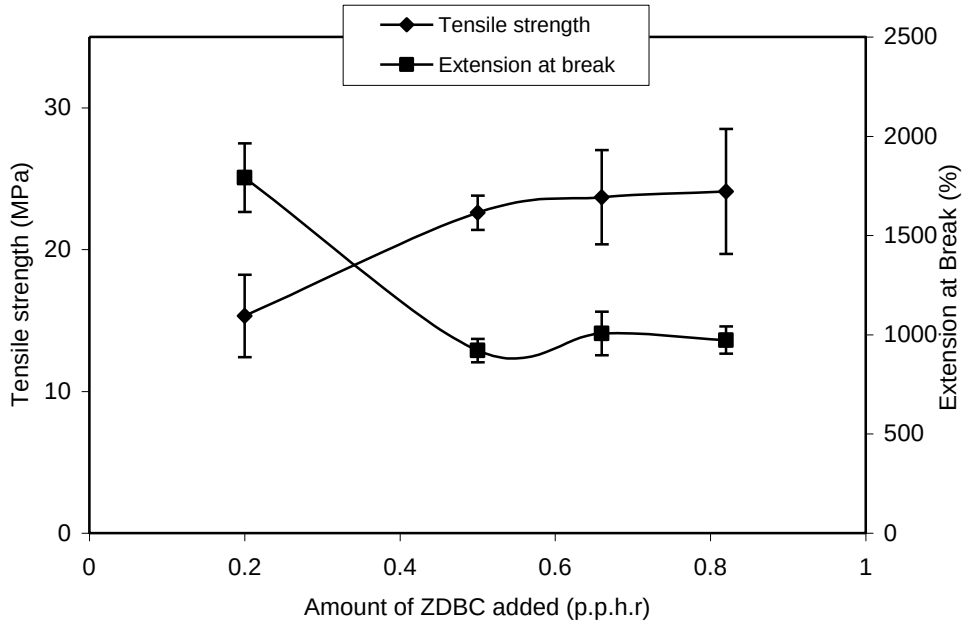


Figure 6: Effect of varying amount of ZDBC on tensile strength and extension at break

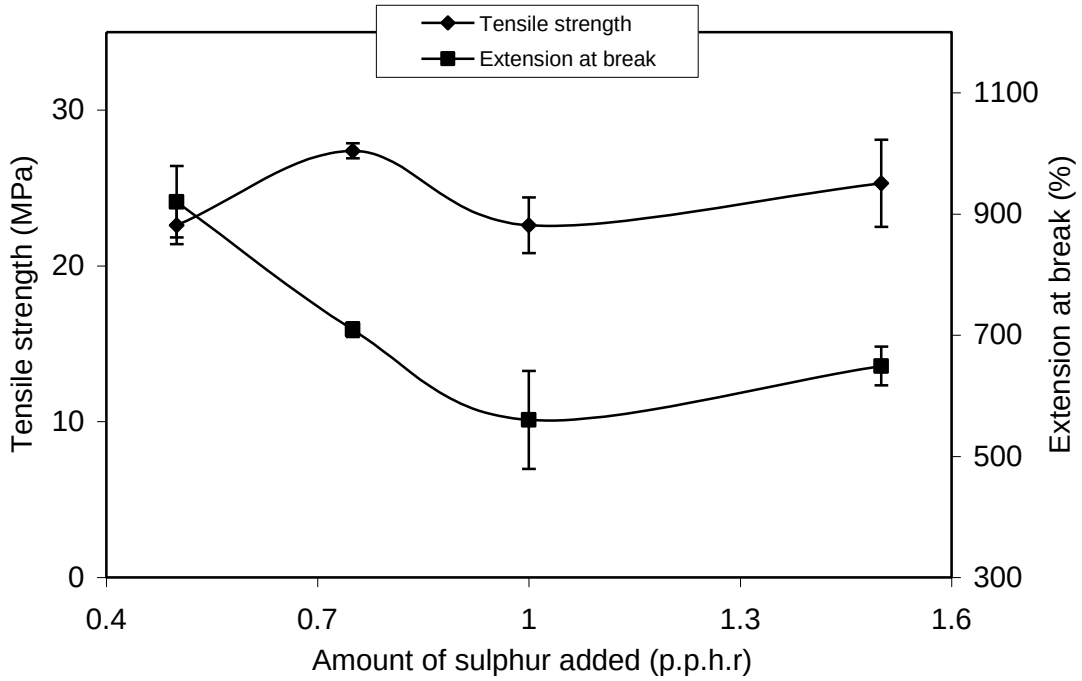


Figure 7: Effect of varying amount of sulphur on tensile strength and extension at break

Figures 6 and 7 showed that an acceptable level of the tensile strength and extension at break as required by ASTM D 3577-00 was obtained for latex vulcanizates using formulations BU-3, BU-4 and BU-5.

The ZBEC is found to be the least active among the three dithiocarbamates. Figure 8 shows the variation in tensile properties of latex vulcanizates with increasing level of ZBEC. It was found that increasing the ZBEC did not impart sufficient tensile strength to the latex sheets (figure 8). The desired level of tensile properties was achieved in presence of a secondary

accelerator, namely, ZMBT. This was, however, achieved at a higher loading of sulphur of 1phr (figure 9).

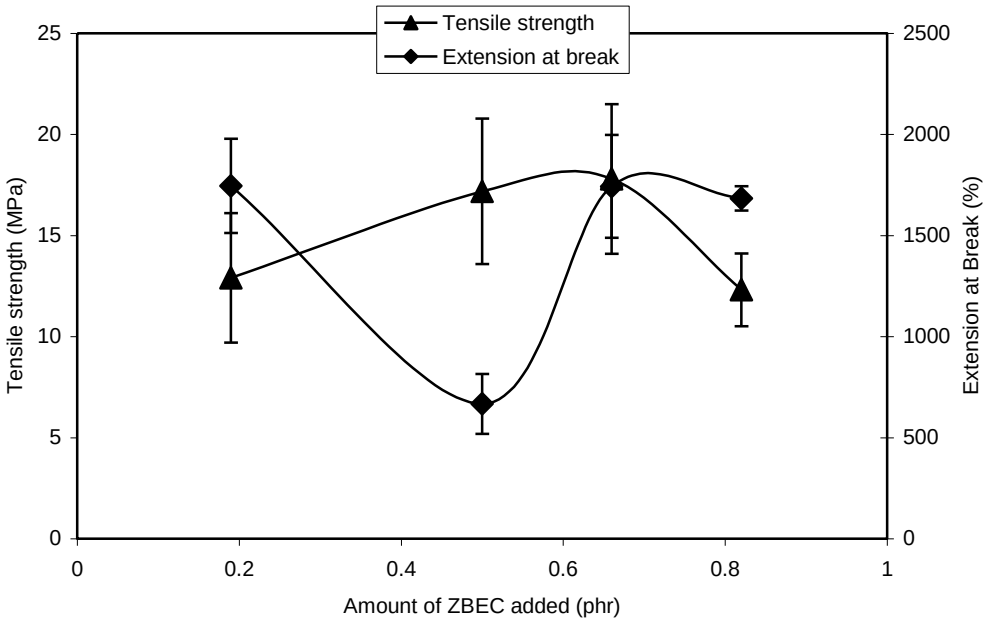


Figure 8: Effect of varying amount of ZBEC on tensile strength and extension at break

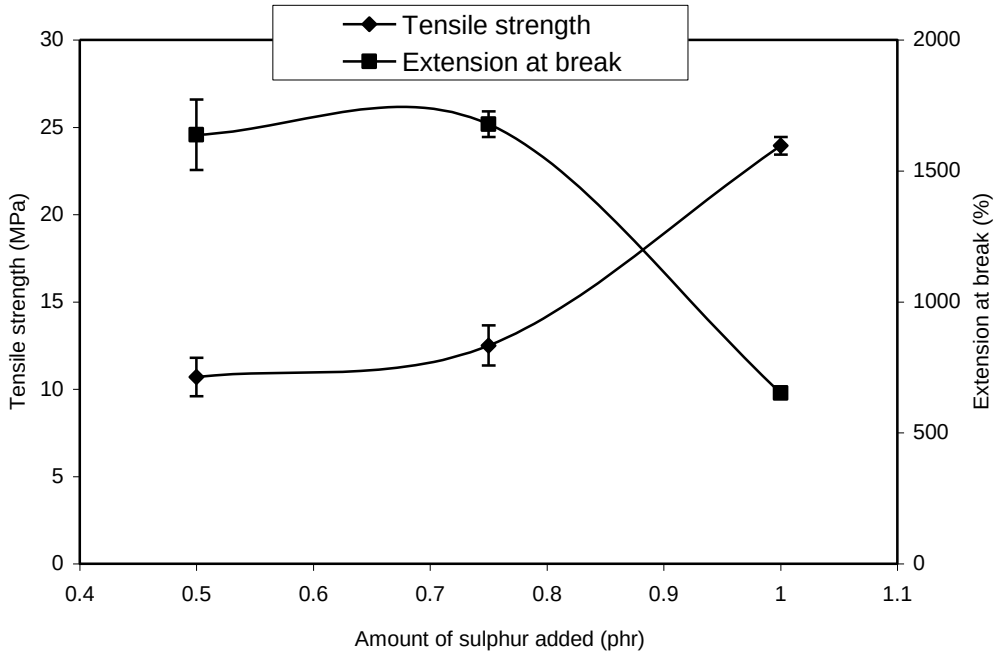


Figure 9: Effect of varying amount of sulphur on tensile strength and extension at break in presence of ZMBT

The results of the tensile properties of the latex vulcanizates using ZDEC following oxidative ageing is given in Table 9.

Table 9: Effect of ageing on the mechanical properties of latex sheets

	Tensile strength (MPa)		Elongation at break (%)	
	Before ageing	After ageing	Before ageing	After ageing
DE-12	25.3 ± 1.77	19.9 ± 1.5	738 ± 21	652 ± 15
DE-12AO	22.2 ± 0.97	17.9 ± 1.5	654 ± 12	683 ± 35

It was found that the tensile properties of aged vulcanizates met the requirements put forward by the ASTM D 3577-00 with regards to the surgical and examination gloves. For surgical gloves, ASTM specifies a minimum value of 24 MPa and 18 MPa for tensile strength before and after ageing respectively. With regards to the elongation at break, the glove requires a minimum value of 750 and 560% before and after ageing. As far as the examination gloves are concerned, the minimum specified values for tensile strength and elongation at break are lesser than that specified for surgical gloves. In the present case, the tensile strength of one of the final formulations before and after ageing was 25.3 MPa and 19.9 MPa respectively. Its percentage elongation at break before and after ageing was 738 and 652 respectively.

Estimation of DCM-extractable ZDEC

Figure 10 shows the effect of varying level of sulphur on the amount of DCM-extractable ZDEC while maintaining a constant level of ZDEC in natural rubber latex vulcanizates.

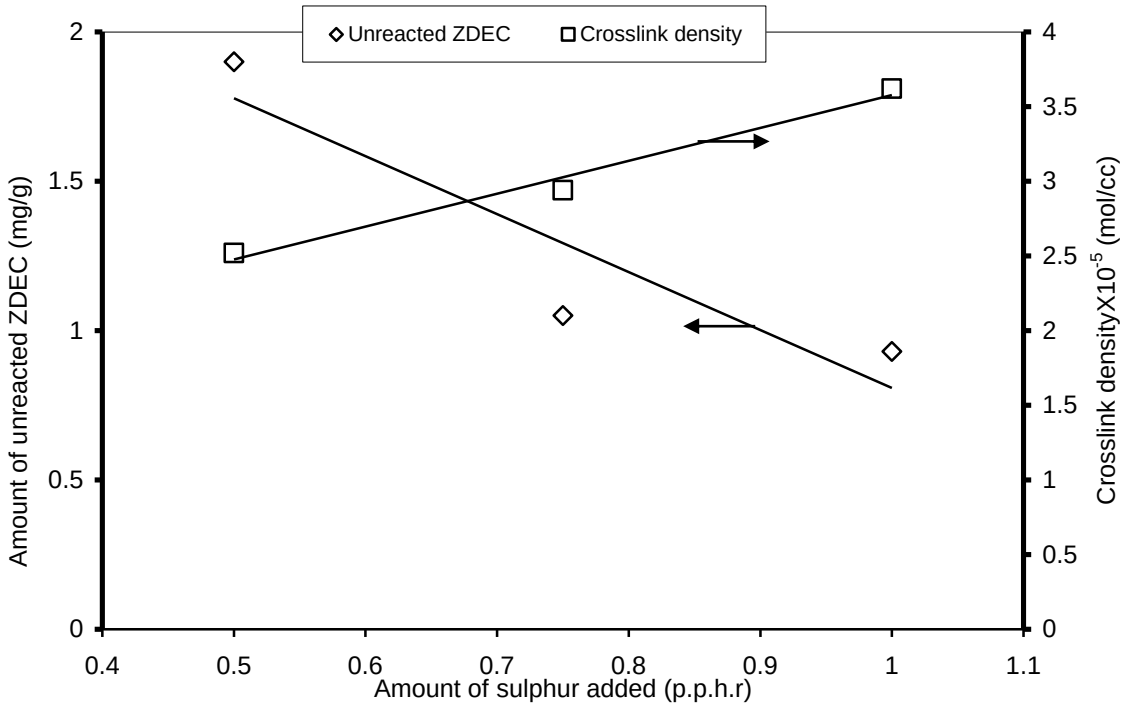


Figure 10: Effect of varying amount of sulphur on DCM-extractable ZDEC and crosslink density

It was observed that increasing the sulphur level while maintaining a constant ZDEC content of 0.5 p.p.h.r tends to increase the crosslink density and decrease the DCM-extractable ZDEC. The decrease in the amount of DCM-extractable ZDEC in the vulcanizates may be attributed to the effective utilization of ZDEC in the network formation at higher levels of sulphur. However, the amount of solvent-extractable ZDEC did not vary significantly when the level of sulphur was

0.75 and 1.0 p.p.h.r. At these sulphur levels, only about 20% of the added ZDEC was extracted into the dichloromethane from the latex vulcanizates. On the other hand, as high as 38% of the added ZDEC was extracted when the amount of sulphur was 0.5 p.p.h.r.

Estimation of sweat-extractable ZDEC

The effect of varying amounts of sulphur on the sweat-extractable ZDEC is shown in figure 11.

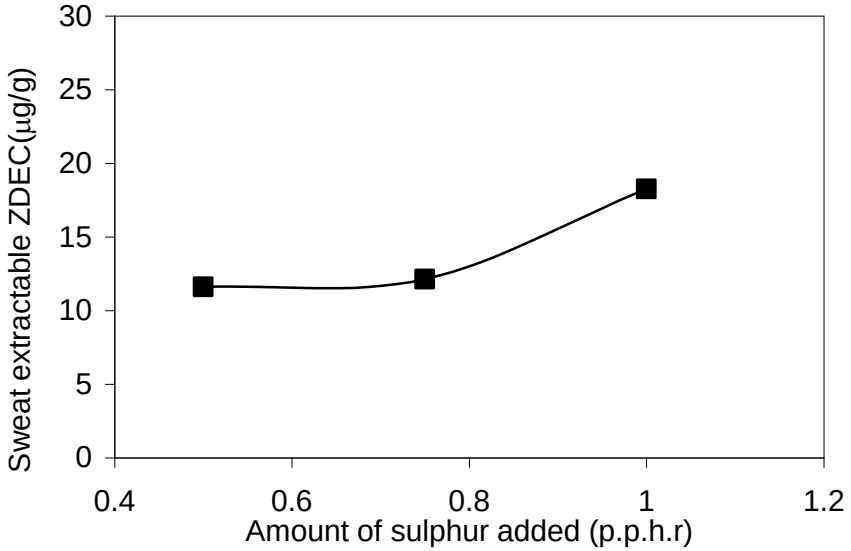


Figure 11: Effect of varying amount of sulphur on sweat-extractable ZDEC

The sweat-extractable ZDEC, in contrast to the DCM-extractable ZDEC, showed no significant variation with an increase in the sulphur concentration except when a 1 p.p.h.r was used. The results, in general, indicated that the migration of ZDEC residues into the artificial sweat is limited to the surface of the vulcanizates.

Alkaline wash of vulcanized latex sheets

The effect of alkaline wash on the amount of ZDEC migrating into artificial sweat was shown in Table 10.

Table 10: Effect of alkaline wash on sweat-extractable ZDEC

Sample	Sweat-extractable ZDEC (μg/g)
DCE-12 (Before alkaline washing)	12.18
DCE-12 (After alkaline washing)	0.28

The alkaline washing caused a dramatic reduction in the amount of residual ZDEC from the surface of vulcanized sheets by 98% indicating the alkali washing may be used as an effective method to remove the residual ZDEC from latex products. The effect of alkaline washing on the tensile properties of the latex vulcanizates was evaluated and the results are given in Table 11.

Table 11: Effect of alkaline wash on the tensile properties of latex vulcanizates

Formulation codes	Tensile strength (MPa)		Extension at break (%)	
	Before alkaline wash	After alkaline wash	Before alkaline wash	After alkaline wash
DCE-12	25.3 ± 1.77	22.6 ± 1.95	738 ± 21	698 ± 50
BU-5	27.38 ± 0.48	27.2 ± 2.3	709 ± 12	643 ± 17
DE-12AO*	22.2 ± 0.97	17.2 ± 1.7	654 ± 12	739 ± 6

(*- samples that were subjected to ageing)

The reduction in the tensile strength and extension at break of the vulcanizates was only marginal and the percentage decrease was found to be below 10% in the all formulations studied. It was also observed that the decrease in the tensile properties of aged latex vulcanizates following alkaline wash was within the limit specified by ASTM D3577-00.

9.2.4 Biological Characterization

9.2.4.1 *In vitro* cell culture cytotoxicity test

The latex vulcanizates using different dithiocarbamate accelerators were subjected to *in vitro* cell culture cytotoxicity test. They showed moderate to severe cytotoxicity response to L929 cells (Table 12).

Table 12: I*n vitro* cell culture cytotoxicity response to various latex vulcanizates in direct contact test

Code	Direct Contact test
DCE-1	Severely cytotoxic
BU-7	Moderately cytotoxic
BU-1	Severely cytotoxic
BZ-2	Severely cytotoxic

As the latex vulcanizates showed cytotoxicity response, the leaching process was modified to reduce the residual dithiocarbamates in the latex vulcanizates. An additional leaching in alkali solution to remove the residual dithiocarbamates adhered to the surface of the latex sheets was given. The latex vulcanizates were again subjected to in vitro cell culture analysis to assess the toxicity towards L929 cell lines. The results are shown in Table 13. Both the vulcanizates using ZDEC (DCE-12 and DCE-12AO) was found to be non toxic. However, the vulcanizate cured with ZDBC showed a moderate cytotoxic response in spite of giving an alkali leaching.

Table 13: I*n vitro* cell culture cytotoxicity response to various latex vulcanizates

Code	Direct Contact test	Test on extract
DCE-12	Non cytotoxic	Non cytotoxic
BU-1	Moderately cytotoxic	Moderately cytotoxic
DCE-12AO	Non cytotoxic	Non cytotoxic

Figure 12(a-c) shows the typical cytotoxicity response to negative control, positive control and DCE-12 in direct contact test. A similar response was obtained in the test on extract assay for DCE-12. The latex vulcanizate using formulation DCE-12AO also exhibited a similar response in both direct contact and test on extract.

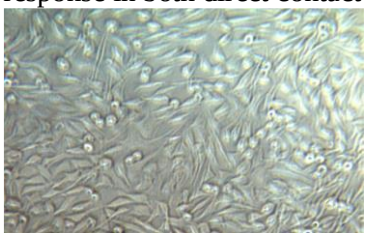


Figure 12a: L929 cells incubated with negative control

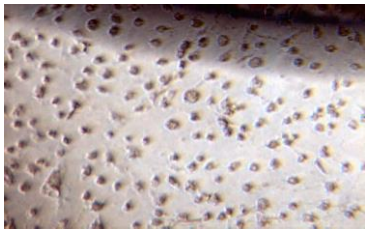


Figure 12b: L929 cells incubated with positive control

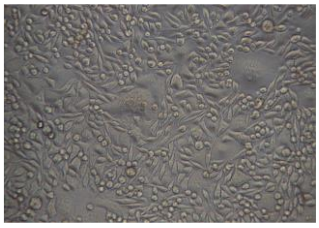


Figure 12c: L929 cells incubated with DCE-12

The cells retained their spindle shaped morphology in direct contact and test on extract assay indicating that both DCE-12 and DCE-12AO vulcanizates were non toxic to the L929 cells.

Hemolysis

The average hemolytic index of DCE-12 is given in Table 14.

Table 14: Hemolytic index of DCE-12

Type of test	Sample code	Direct/extract method	Hemolytic index
Static test	DCE-12	Direct	0.74 ± 0.035
		Material Extract	0.692 ± 0.026
	Negative control	Direct	0.569 ± 0.045
		Material Extract	0.202 ± 0.01
Dynamic test	DCE-12	Direct	0.59 ± 0.021
		Material Extract	0.77 ± 0.0058
	Negative control	Direct	0.491 ± 0.074
		Material Extract	0.317 ± 0.019

The results indicated that, under static condition, the material (DCE-12) induced an average hemolytic index of 0.74 ± 0.035% in direct contact method and 0.692 ± 0.026% in extract method. Under dynamic condition, DCE-12 induced an average hemolytic index of 0.59 ± 0.021% in direct contact method and 0.77 ± 0.0058% in extract method. The average hemolytic index of DCE-12AO is given in Table 15.

Table 15: Hemolytic index of DCE-12AO

Type of test	Sample code	Direct/extract method	Hemolytic index
Static test	DCE-12AO	Direct	0.453 ± 0.031
		Material Extract	0.409 ± 0.036
	Negative control	Direct	0.569 ± 0.045
		Material Extract	0.202 ± 0.01
Dynamic test	DCE-12AO	Direct	0.409 ± 0.02
		Material Extract	0.282 ± 0.03
	Negative control	Direct	0.491 ± 0.074
		Material Extract	0.317 ± 0.019

The results indicated that, under static condition, the material (DCE-12AO) induced an average hemolytic index of 0.453 ± 0.031% in direct contact method and 0.409 ± 0.036% in extract method. Under dynamic conditions, DCE-12AO induced an average hemolytic index of 0.409 ± 0.02% in direct contact method and 0.282 ± 0.03% in extract method.

Intracutaneous irritation studies

The intracutaneous irritation score for DCE-12 and DCE-12AO are given in Table 16& 17.

Table 16: Intracutaneous irritation score for DCE-12

Animal	Primary irritation score							
	Erythema				Oedema			
	Saline	CSO	PEG*	Alcohol Saline*	Saline	CSO	PEG*	Alcohol Saline*
Animal 1	0	0.2	0	0	0	0	0	0
Animal 2	0	0	0	0	0	0	0	0
Animal 3	0	0	-	-	0	0	-	-

**- test was performed according to ISO 10993-10: 2002 (E); PEG-Polyethylene Glycol*

Table 17: Intracutaneous irritation score for DCE-12 AO

Animal	Primary irritation score									
	Erythema					Oedema				
	Saline	CSO	Artificial sweat*	PEG*	Alcohol:Saline*	Saline	CSO	Artificial sweat*	PEG*	Alcohol Saline*

Animal 1	0	0.7	0	0.2	0	0	0	0	0	0
Animal 2	0	1.1	0	0	0	0	0	0	0	0
Animal 3	0	0.7	-	-	-	0	0	-	-	-

*- test was performed according to ISO 10993-10: 2002 (E)

The primary irritation index of the material tested is given in the Table 18.

Table 18: Primary irritation index of DCE-12 and DCE-12AO

Material code	Primary irritation index				
	Saline	CSO	Artificial sweat	Polyethylene Glycol 400	Alcohol Saline
DCE-12	0	0.02	NT	0	0
DCE-12 AO	0	0.28	0	0.016	0

NT-not tested

The primary irritation index for both the test specimens in physiological saline , Alcohol Saline and artificial sweat was zero. On the other hand, the primary irritation index of DCE-12 and DCE-12AO respectively in cotton seed oil was 0.02 and 0.28 and on PEG was 0 and 0.016.

Sensitization test

Tables 19 & 20 give the response to closed patch sensitization studies for erythema and oedema of the control and test materials.

Table 19: Responses to closed patch sensitization test recorded for DCE-12 for test and control animals (challenge application)

AnimalNo.	Group	Skin reaction			
		Erythema		Oedema	
		24 h	48h	24h	48h
1	Test	0	0	0	0
2	Test	0	0	0	0
3	Test	0	0	0	0
4	Test	0	0	0	0
5	Test	0	0	0	0
6	Test	0	0	0	0
7	Test	0	0	0	0
8	Test	0	0	0	0
9	Test	0	0	0	0
10	Test	0	0	0	0
11	Control	0	0	0	0
12	Control	0	0	0	0
13	Control	0	0	0	0
14	Control	0	0	0	0
15	Control	0	0	0	0

Table 20: Responses to closed patch sensitization test recorded for DCE-12AO for test and control animals (challenge application)

AnimalNo.	Group	Skin reaction			
		Erythema		Oedema	
		24 h	48h	24h	48h
1	Test	0	0	0	0
2	Test	0	0	0	0
3	Test	0	0	0	0
4	Test	0	0	0	0
5	Test	0	0	0	0

6	Test	0	0	0	0
7	Test	0	0	0	0
8	Test	0	0	0	0
9	Test	0	0	0	0
10	Test	0	0	0	0
11	Control	0	0	0	0
12	Control	0	0	0	0
13	Control	0	0	0	0
14	Control	0	0	0	0
15	Control	0	0	0	0

For all observation period the numerical grading for erythema and oedema for test specimens was zero.

CONCLUSIONS

The present work facilitated the development of non-toxic natural rubber latex formulations using ZDEC suitable for medical applications such as gloves. The latex vulcanizates DCE-12 & DCE-12AO showed adequate tensile properties before and after aging as specified in ASTM D 3577-00 for gloves. The artificial sweat extraction studies of the latex vulcanizates indicated that negligible quantities of residual ZDEC migrated into the sweat solution compared to the commercial brands of gloves tested. The removal of residual dithiocarbamates from the surface of the latex vulcanizates is facilitated by subjecting them to an alkaline washing in addition to water leaching.

The in vitro cell culture cytotoxicity assay showed that the vulcanizates were toxic to cell lines. However, on improving the leaching process, the latex vulcanizates using formulations DCE-12 & DCE-12AO showed a non toxic response to L929 cells. The material (DCE-12 and DCE-12AO) was found to be non hemolytic. Tissue response to intracutaneous injections from the material extracts of DCE-12 and DCE-12 AO showed that the material did not elicit erythema or oedema. The sensitization studies of DCE-12 & DCE-12AO revealed that the material does not elicit any immune response in guinea pigs.