Discrimination_and_Cel_Counting_of_Superoxide_Dismutase_SOD.pdf

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Submission date: 15-Jun-2021 10:18AM (UTC+0700)
Submission ID: 1606681985
File name: Discrimination_and_Cel_Counting_of_Superoxide_Dismutase_SOD.pdf (1.68M)
Word count: 5634
Character count: 29827

International Journal of Computer Science and Information Security (IJCSIS),
Vol. 14, No. 5, May 2016

Discrimination and cell counting of profile of superoxide dismutase (SOD) under hypercholesterolemia using K-means clustering

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***Abstract*—Clove leaf extract contains high natural antioxidant. In this study, an experiment was conducted to analyse the effect methanol extract of clove leaf on the profile of superoxide dismutase (SOD) in the rabbits liver under hypercholesterolemic condition. Rabbits were divided into three groups i.e (1) negative control group, (2) positive control (hypercholesterolemic) group, which fed diet containing 1% cholesterol for 50 days, and (3) group which was given clove leaf extract and 1% cholesterol simultaneously for 50 days. Contents profile of antioxidant superoxide dismutase in rabbit liver tissue was obtained using immunihistochemical techniques. The images were taken from each group using a microscope to analyse the reaction of clove leaf extract to the cells. The images were processed using K-mean clustering to discriminate the cells after treatment. The cell segmentation was performed to count number of the cells based on their treatment. The cell counting technique then compared with the manual counting. The results demonstrate that the developed technique was a reliable image processing technique which could be used in the cell counting purposes.**

I. Introduction

Hypercholesterolemia indicates the presence of high level of cholesterol in the blood stream of animals. Cells in the liver are discoloured and become damaged which can be used as an aid to observe the effects of a treatment against hypercholesterolemia.

Cell counting plays important role in the biomedical study. Typically, this task can be done manually by human. However, recognizing objects in the images is becoming interest area in the biomedical subject. Diagnostic pathologists are inclined to shift to automated systems which can aid in diagnosis and help steer clear of laborious tasks [1]. The use of image processing in the cell counting process involves a task called segmentation. Segmentation is a process to group objects or boundaries which have similar characteristics. In the biomedical study, segmentation is usually employed to segment the object of interest within an image for clinical or medical diagnosis. There are a great number of related works on differential cell count. However, little literature exists on automated segmentation and classification of rabbit liver cell under Hypercholesterolemia, whose blood smear sample proposes different set of challenges upon processing.

Several techniques and approaches of segmentation have been proposed to perform cell analysis. [2] utilized gray images of blood smear to segment and classify White Blood Cells (WBCs) into 5 classes. The cells are localized using Hierarchical thresholding with nuclease and cytoplasm extracted as its features. An accuracy of 90% was observed when processing on 71 cells. However the state of cell (healthy or damaged) was not determined. In study [3], edge detection was proposed to detect nuclease in order to count Red Blood Cells (RBC) .The proposed edge operator required a weak edge to be present between the foreground, RBC, and the background which was often unsatisfied. Using similar method, [4] analysed histological sections from different squamous cell cancers and initially counted all the cells, irrespective of their nature. Using Principal Component Analysis (PCA) along Optimum Laplacian of Gaussian Assimilator (OLGA), the stained cells are then segmented at a certain threshold value. The performance of this technique is weaker when two or more cells of similar colour intensity are overlapped and are erroneously counted as single cell. [5] exploited the feature of geometry of White Blood Cell (WBC). Cells were localized and counted based on their shape. However, this technique was only useful in cases where WBCs were circular in shape. [6] obtained segmentation of WBCs from coloured blood smear images that contained both immature and peripheral cells. A 57 dimensional feature vector was developed from various features including shape, texture and colour. This technique incorporated the use of morphological pre-processing followed by segmentation through fast snake balloon method. Different classifiers were adopted, and a peak accuracy of 91% was obtained using Support Vector Machines (SVM). However, the health condition of cells has not been mentioned. [7] suggested segmentation of WBC from bone marrow, using Granulometric

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moments, area and location of Nuclease as feature vector. Bayesian classifier was used for classification to yield an accuracy of 77% on the data set. [8], [9] demonstrated a parameter-free algorithm called Watershed technique which interprets the grey-level in the image as its variation of topographic reliefs. However, Watershed technique often suffers from over-segmentation problem [10]. [11], [12] use Active Contour for the detection of objects in an image. The method uses a boundary (called snakes) around the object. The boundary moves toward the object based on the minimization of energy function for segmentation process. This technique is dependent on the assignment of first contour and occasionally fails to follow the contours of original cells correctly. Previous studies have also reported application of K-means clustering algorithm for segmentation in the biomedical. [13] demonstrated the use of this technique to segment 3-D image data of CT volumetric images. In an investigation on skin diseases, [14] conducted a study to detect psoriasis objects in color-skin images.In such cases K-means clustering has advantages of being simple, fast and flexible [15], [16].

It is noteworthy from the review of the studies that an assortment of colour, position, edge and shape makes cell segmentation a challenging task in medical image processing. An algorithm that achieves high accuracy for a certain cell segmentation problem may not be an effective solution for another problem due to variation in characteristics and features of cells. Hence, different techniques may be adopted for different problems to achieve similar accuracy level.

In this study, three groups of rabbits were treated with different treatments using clove leaf extract. Extract from clove leaf (Eugenia aromatica) contains higher potential antioxidant than standard antioxidant compound butylated hydroxytoluene (BHT) [17], [18]. However, due to challenging nature of the research problem, researchers have not explored the influence of the clove leaf as an antioxidant and antihypercholesterolemic to a rabbit.

The reaction of the liver cells to antioxidant from clove leaf extract generated different colours on the cell surfaces. Each colour on the cell surface describes the health condition of the cells. K-means clustering was adopted to segment cells utilizing its colours and Euclidean distance as features. For the mentioned task, the technique significantly reduces the problem of occlusion, under segmentation and over segmentation. The proposed cell counting technique was applied to facilitate an accurate and reliable cell counting in the liver of rabbits. The technique combines different existing image processing algorithms to obtain a powerful technique for cell counting.

Extract from clove leaf (Eugenia aromatica) contains potential antioxidant higher than standard antioxidant compound butylated hydroxytoluene (BHT) [17], [18]. However, much of the researchers up to now have not explored the influence of the clove leaf as an antioxidant and antihypercholesterolemic to a rabbit. In this study, three groups of rabbits were treated with different treatments using clove leaf extract. The purpose of the treatments is to analyse the effect of the clove leaf extract to the cholesterol that had been fed to rabbits. The reaction of the liver cells to antioxidant from clove leaf extract generated different colours on the cell surfaces. Each colour on the cell surface describes the condition of the cells. The proposed cell counting in this paper was applied to facilitate an accurate and reliable cell counting in the liver of rabbits under hypercholesterolemic condition after treatment using clove leaf extract. The technique derived from different existing image processing techniques but it combines together to obtain a powerful technique for cell counting.

II. Material and method

A. Preparation of extracts

Clove leaves were left to dry and 15 gram of them were sampled and chopped using a blender. The chopped clove leaves were finally mixed with methanol, ethanol and distilled water. Extraction was performed by reflux for 6 hours and then filtered. The filtration result was evaporated until 6 ml using vacuum rotatory evaporator at 45 ^{o}C. The extraction result was keep in the freezer at -10 ^{o}C.

B. Sampling method

9 male New Zealand Rabbits weight about 3-4 kg were obtained from Agricultural and Livestock Service, Bogor, Indonesia. They were adapted to the local environment for a month and grouped into 3 treatments and each group consists of 3 rabbits. Each rabbit had its own special cage for rabbits. Animal experiments were conducted in accordance with Indonesian national guidelines for the use and care of animals on health research and were approved by local Animal Care Committee of State University of Makassar . Rabbits weight about $\pm$2-3 kg were used in this study. They were adapted to the local environment for a month and grouped into 3 treatments and each group consists of 1 rabbit. First group is negative control group (K-). This group was fed with standard ration. Second group is positive control group/hypercholesterolemia (K+). The group was fed with standard ration and cholesterol. The cholesterol is given by 1% from the average weight of ration consumed by the rabbit. Third group (EDC+K) is group which was given clove leaf extract (1 g/kg-bw/day) and cholesterol 1% of average weight for 50 days. In the adaptation period, the rabbits consume ration about 100 g/head/day, so the amount of cholesterol that is added is 1% of 100 g or 1 g/head/day. Cholesterol was given by mixing 1 g of cholesterol crystals into a small ration (30 g). 50 days after treatment, anesthesia was induced to all rabbits using atropine 1-3 mg/kg by intramuscular injection and 30 minutes after that, diazepam (valium) 0,5-5 mg/kg was injected intramuscularly. The liver off all rabbits were excised and liver tissue samples from each of the rabbits were separated and prepared using the Paraffin technique for the analysis of SOD activity and the content of Cu and Zn-SOD levels. The experiment method was adopted from our previous study [19].

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C. Image Acquisition

The images were taken using a microscope at a magnification of 400 and scale of 50 μm. The experiment conducted twice in order to achieve data a and b. There were two experiments prevention (P) and cure (C) each after 10, 20 and 30 days. The effect of experiments were to be reaffirmed through visual processing techniques. The files were named based on the experiment for instance, C20 b means cure experiment after 20 days for data b.

The section of image processing and analysis of this study is commenced by acquiring the images of tissues samples. The resolution of images obtained was 2048 x 1536 pixels. Since the study does not require the details of samples at individual pixel level, the test images were down sampled by a fraction of four. The resultant resolution was 512 x 384 pixels which favoured the course of study by reducing the processing time significantly, yet maintaining the required data.

D. Cell counting methodology

Digital Image Processing can provide more efficient result as compared to tradition technique of counting different cells manually. This technique is not only fast and accurate but also reliable. Multiple shades of healthy and damaged cells can be categorized into respective sets depending upon shape, size, distance and colour [20]. Figure 1 illustrates the basic outline of the operations performed in this study.

Pre-processing serves as the second step in the course of achieving our objective. Image pre-processing techniques are employed in order to obtain better result. It generally comprises of a combination of different processes depending upon the desired result. Here the image was pre-processed using two operations in order to improve the chances of success in image segmentation. Firstly a Median filter of large kernel size was applied on the image. If a set X of random variables is given in an ordered fashion. Then a median value can be obtained as :

$$median(X) = \begin{cases} X_{(K+1)} = X_{(m)} & forN = 2K+1 \\ 1/2X_{(K)} + X_{(K+1)} & forN = 2K \end{cases} \quad (1)$$

In the above equation $m = 2k + 1$ is rank that includes median value. Median filter first sorts the values in increasing order and then replaces the value at beginning of kernel with its median value. A two-dimensional filter for applying on a grey scale image can be obtained as :

$$Y_{i,j} = \frac{median}{(r,s)\epsilon W} (X_{i+r,j+s}) \quad (2)$$

Where r ands belong to W, which is a region on which the filter is applied. Median filter is very powerful in removing scattered noise from image. It removes noise quite effectively when applied on the image. The second objective was to create a smoothing effect in the image so that the cells could easily be separated as a whole body upon segmentation [21]. Since the median filter is defined for 2-Dimensional single channel image, therefore, it was applied on all three colour channels separately. The results were combined to produce an overall effect of median over a colour image. Figure 2 illustrates the use of median filter on an image.

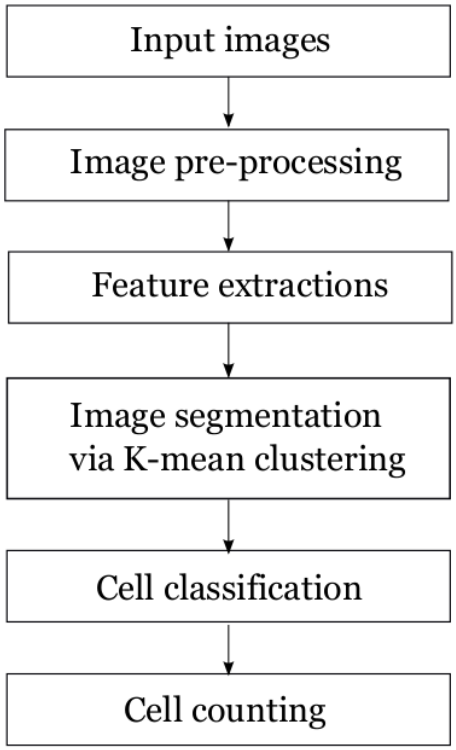

Fig. 1. Proposed image processing technique

The next step in pre-processing was to use Adaptive Histogram Equalization [22] (AHE) in order to improve the chances of successful segmentation. It improves the contrast level in different sections of the image. AHE is different from conventional Histogram equalization (HE) in the sense that AHE computes multiple histograms. Each histogram is obtained corresponding to a distinct small region of the image, which aid to distribute the contrast level of the entire image. AHE treat regions in segregated manner and thus improves the local contrast of the image [23]. The result is a more finely detailed image. The effect of HE and AHE, when applied on the previously filtered (median) image could be seen in Figure 3

The goal of this study was to observe the response of cells under hypercholesterolemia to our treatment and to monitor their health. In response to the treatment, the healthy cells maintain their shades of blue colour while the damaged cells turned brownish or purple. An essential step before conducting further experimentation was to extract relevant features based on which the cells could be segmented. These cells would

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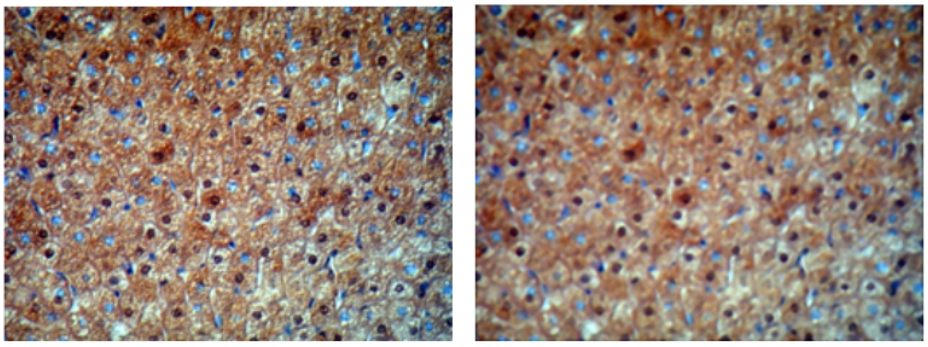
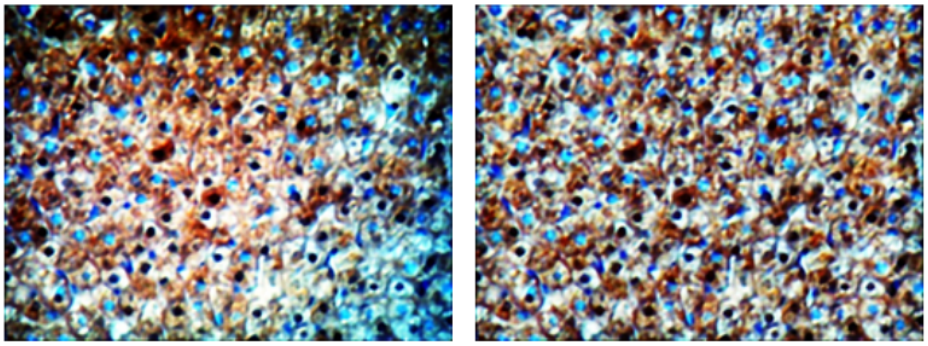


(a) (b)

Fig. 2. Result of median filter on an image (a) Original rabbit liver sample (b) resultant



(a) (b)

Fig. 3. The effect of two different Histogram equalizations (a) Resultant of Histogram Equalization (b) Resultant of Adaptive Histogram Equalization

serve as the foreground that would be subtracted from the background. The issue was that they vary both in sizes and shapes. Some of the cells are completely rounded while other may be elliptical or almost rectangular. Similarly some of the cells also vary in size. In order to achieve best possible outcome and avoid erroneous result, a common feature must be selected among all cells, based on which they could be classified. For this study, the variation in colour of different cells serves as the most appropriate criterion for segmentation. Since, all cells are different in colour than the background, therefore, extraction of foreground (cells) will be possible with relative ease. Moreover, different cells could be further classified into subgroups based on their colour in order to observe the health of cells.

Clustering and Segmentation techniques were employed in order to separate and categorize regions of an image into clusters, based on their differences or similarities. Clustering is the differentiation and classification of a set of data points into different groups(Clusters). Data points with similar feature vectors are placed in the same cluster. The measure of difference between two feature vectors can be obtained using Euclidean or Mahalanobis distance [24]. K-means Clustering is a well-developed and widely accredited technique to partition multi-dimensional data into K- clusters. The resultant function of K-means clustering achieves an optimized distribution of feature vectors based on their similarities and distance measures.

In order to incorporate the effect of colour of cells during clustering, image was first converted from RGB colour space to L*a*b* colour Space. The L*a*b* space consists of three layers (i) Luminosity layer 'L*' (ii) Chromaticity-layer 'a*' and (iii) Chromaticity-layer 'b*' .The chromaticity-layers indicate where colour falls along the red-green axis, blue-yellow axis, for a* and b* respectively. Now, we have all the colour information in the 'a*b*' space, where objects (cells) are pixels with certain values of a*' and 'b*' without any involvement of non-chromatic factors such as brightness. These pixels constitute the dataset which will be clustered and segmented using K-means algorithm.

In this study we utilized MATLABs built-in function for K-means clustering. MATLAB uses k-means++ algorithm [25] in order to initialize cluster centres (or seeds), where the first centroid c_1 is chosen at random from the dataset X of N points/pixels $(x_1, x_2, , x_n)$. For each data point, we calculate the squared Euclidean distance to the previously selected

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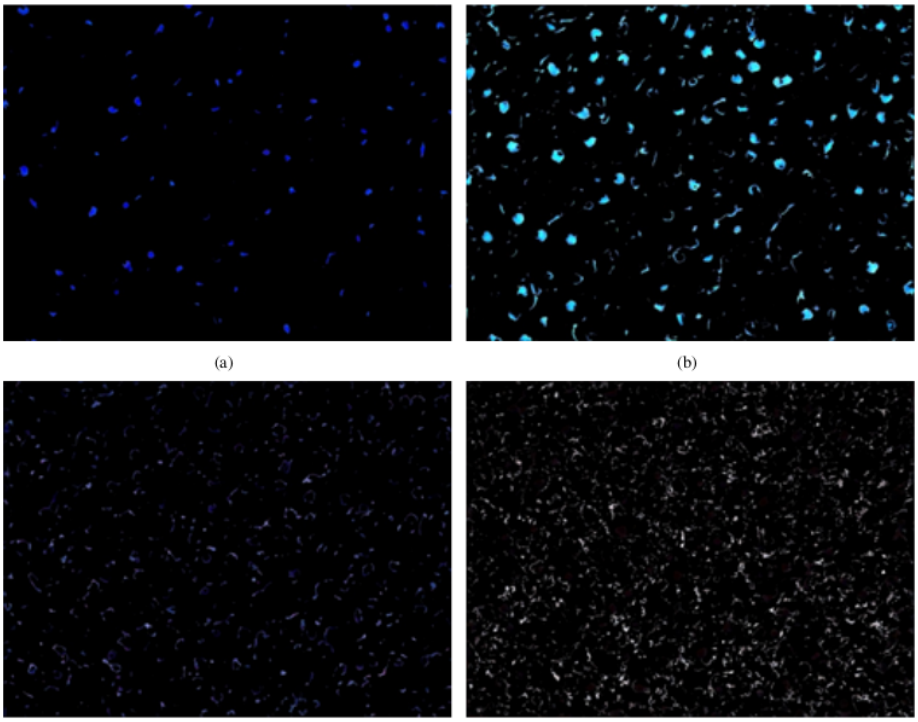

Fig. 4. Relevant segments extracted through segmentation using K-means (a)Segment 1 pertaining to Healthy Cells (b) Segment 2 pertaining to Healthy Cells (c) Segment 3 pertaining to Damaged Cells (d) Segment 4 pertaining to Damaged Cells

centroid through equation 3. This shortest distance is denoted by d_i.

$$d_i^2 = ||x_i - c_1||^2 \tag{3}$$

Then the subsequent centroid c_k is obtained using the probability function given in equation 4. Here each distance d_k is marked from the previous centroid (c_1 , for first step) to the observations/pixels.

$$\frac{d_i^2}{\sum_{k=1}^{N} d_k^2} \tag{4}$$

The distance d_i is recomputed followed by introduction of a new centroid c_2 with the above stated probability function. An extension of equation 3 gives the new value of d_i by using equation 5.

$$d_i^2 = min||x_i - c1||^2, ||x_i - c_2||^2 \tag{5}$$

This process is repeated until K centroid are obtained, which marks the end of appropriate seeding through k-means++ algorithm. This point onwards standard K-means algorithm is used to assign observations/pixels to the cluster C_i , where C_i is the set of points from dataset X that are closer to centroid c_i.This iteration continues until the algorithm converges and no further changes are observed in clusters C_i.

Various tests were conducted at different values of K (number of clusters) in order to observe the response of K-means. Segmentation was performed in two stages in order to achieve the most adequate result [26]. At K=2, the foreground (cell colours and similar shades) was segmented from the brown background of the cell image. This step helps to avoid large value of K that is required due to clustering and segmentation of the irrelevant shades of brown background. In the second step, the foreground image was sub-segmented at K=20 to extract four different colours. Segments with healthy cells contained Cyan and Blue colour while damaged cells had Brown and Bluish Purple colour. The four relevant segments are shown in Figure 4.

Some images may produce different result after segmentation due to variation in illumination and level of intricate data that needs to be extracted. Even after dedicated pre-processing, efficient segmentation might not be achieved and often the cell segments into two or more clusters. The image segment that contains such cell fragments is identified and their portions added back to higher class/category. During experimentation, similar effect was observed in some cases where the cells

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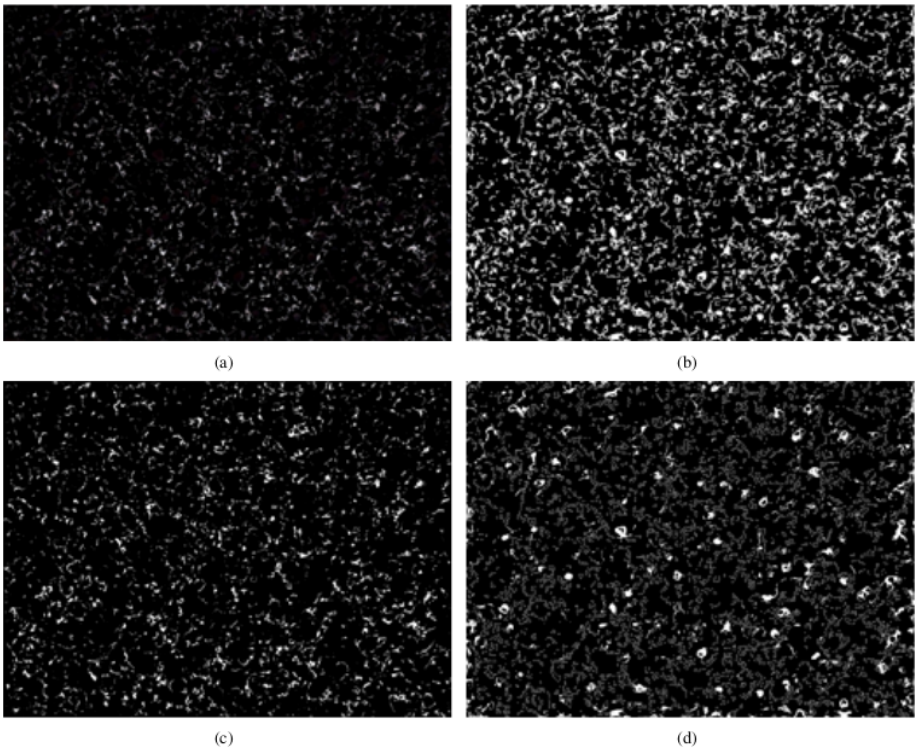

Fig. 5. the use of Thresholding in multiple steps (a) Damaged cell segments with unwanted data (b) thresholded image at value 0.025 (c) thresholded image using Otsuś methods (d) Cells were extracted through the difference of Figure 5a and 5c

were either partially damaged or in a transition towards health deterioration. As a result these calls contained multiple shades of one or more colours.

Thresholding is an effective method to create a distinction between foreground and background by transforming an image to binary format. The first step was converting RGB image to gray scale followed by adjusting the contrast level of image. Binary images allow variety of other processing operations due to much simple form of data. The equation for Thresholding could be defined as: If $f(x, y)$ needs to be thresholded by some value T then $g(x, y)$ is given by

$$g(x, y)) = \begin{cases} 1 & iff(x, y) \geq T \\ 0 & otherwise \end{cases} \quad (6)$$

Image Threshold serves as another stage for image segmentation. A problem which is linked with the use of thresholding is the selection of a global threshold value. Different threshold values will give variant results [27]. Too small or too large threshold value might result in the loss of critical information from the image. In order to extract only the required cells out of previously segmented image, a two-step Threshold methodology has been adopted. For extracting damaged cells, the corresponding image segments were thresholded with a small value of 0.025. As a result all data is retained in the resultant binary image. In the second step Otsuś method was used to obtain the threshold value. The result obtained from second step was subtracted from first to obtain darker parts (Damaged Cells) of the segmented [28]. Figure 5 shows the result of this step.

Morphological operations are very useful in term of achieving a result that has relatively low noise and high accuracy. Three operation of such type have been used each achieving a different goal. First and foremost the segmented image was eroded to in order to break down small parts into even smaller portions. This reduces the size of boundaries as well. In second step morphological opening was performed in order to open any boundaries that are slightly touching. In addition opening also removes any noise created during erosion of image. Finally morphological closing was performed to fill up holes and close boundaries that are near each other [29]. Figure 6 shows all the results respectively.

The final task was to acquire the total count of cells in each health state. This was obtained by calculating the number of boundaries in view of the image using MATLABs built-in function ”bwboundaries()”. Special attention was paid that every cell was isolated from its neighbours and no holes were

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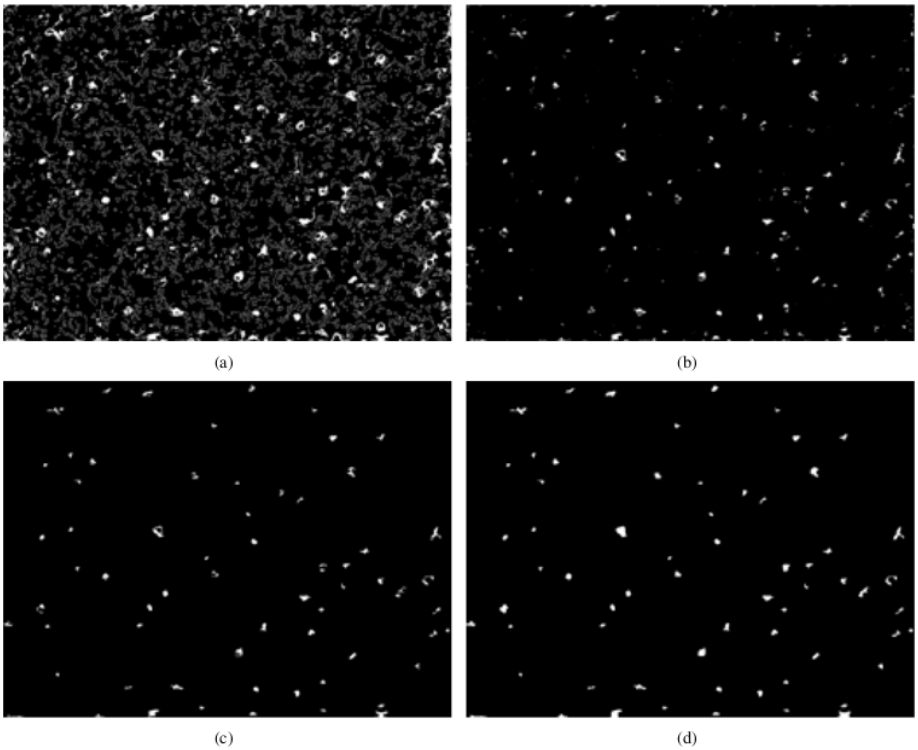
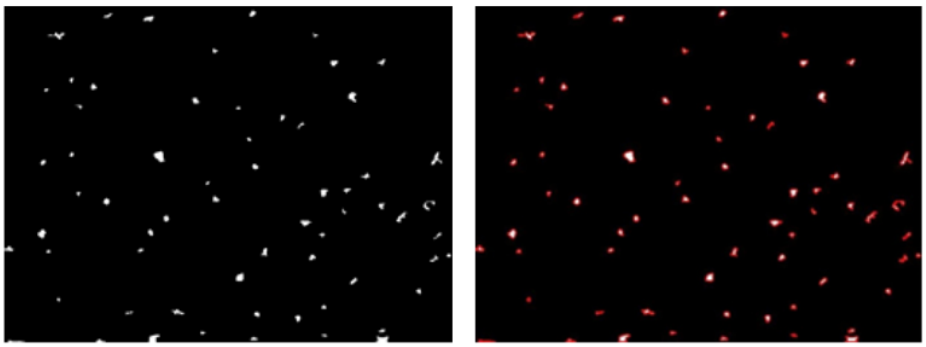


(a) (b)

(c) (d)

Fig. 6. The use morphological operations (a) Thresholded image (b) Erosion performed 5 (c) Morphological Opening (d) Morphological Closing



(a) (b)

Fig. 7. Result of cell counting (a) Image processing Result with cells that needs to be counted (b) Cells counted and boundary colored

present in the object (cell) after segmentation [30]. In case if holes are present then only the outermost boundary of an object is considered for detection, by defining a parameter no holes along the function. All four health states were given distinct boundary colours for purpose of visual demonstration. Figure 7 illustrates the result obtained.

III. Result and Analysis

The results obtained after counting all cells in separate segments are given in tabular form. Table 1 gives the total number of healthy and damaged cells while Table 2 gives a more detailed result representing transition states.

The results of immunohistochemical staining provided different colours on the liver cells which revealed the content of

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TABLE I
RESULT OF THE TOTAL, HEALTHY AND DAMAGED CELLS USING PROPOSED TECHNIQUE AND MANUAL COUNTING (MC) FOR C20 B AND EDC+K (A)

Image Label	Total cells in view / MC	Healthy / MC	Damaged / MC
C10 a	373	257	116
C10 b	417	235	182
C20 a	355	194	161
C20 b	350 / 313	192 / 170	158 /143
C30 a	261	155	106
C30 b	259	155	104
EDC+K (a)	360 /336	176 / 168	184 / 168
EDC+K (b)	328	156	172
K- a	411	156	255
K- b	411	156	255
K- a	425	117	248
K- b	386	181	205
P10 a	520	237	283
P10 b	431	194	237
P20 a	434	228	206
P20 b	425	225	200
P30 a	400	208	192
P30 b	372	170	202

TABLE II
A MORE DETAILED RESULT OF CELL SEGMENTATION FOR STUDYING TRANSITION STATE USING IMAGE PROCESSING AND MANUAL COUNTING (MC) FOR C20 B AND EDC+K (A)

Image Label	Healthy (Cyan) / MC	Healthy (Blue) / MC	Slightly Damaged (Bluish purple) / MC	Heavily Damaged (Brown) / MC
C10 a	187	70	44	72
C10 b	146	89	76	106
C20 a	137	57	44	117
C20 b	110 / 100	82 / 70	54 / 49	104 / 94
C30 a	92	63	N/A	106
C30 b	85	70	12	92
EDC+K (a)	101 / 98	75 / 70	55 / 50	129 / 118
EDC+K (b)	83	73	42	130
K- a	81	75	239	16
K- b	81	75	239	16
K- a	113	64	100	148
K- b	111	70	74	131
P10 a	73	164	93	190
P10 b	93	101	153	84
P20 a	142	86	101	105
P20 b	68	157	100	100
P30 a	57	151	85	107
P30 b	9	161	95	107

Cu, Zn-SOD. The cells colour changed from cyan and blue to brown. Healthy cells were shown by cyan and blue. Brown colour mixed with blue in the hypercholesterolemic group indicates less Cu, Zn-SOD in the liver cells while brown colour indicates more Cu, Zn-SOD in the liver cells.

Manual counting was performed as a comparison to the proposed image processing technique as shown in Table I and II. Experiments C20b and ECD+K(a) were randomly selected. It showed that the result of manual counting is closely related with maximum difference of 11 cells. The average of manual counting is always less than the proposed image processing technique.

IV. CONCLUSIONS

An experiment of rabbit liver reaction to the antioxidant from clove leaves using immunohistochemical techniques has been conducted. The experiment revealed the effect of the antioxidant to the rabbit liver cells under hypercholesterolemia condition which showed by different colour to the cell surface after the antioxidant has been given. A task is needed to detect the antioxidant superoxide dismutase and analyse the number of the cells affected by the antioxidant. The conventional antiquated method that is used for such operation is to detect and count all the cells from images manually. Manual labour is prone to many errors due limitation of human capabilities. The use of dedicated algorithms will reduce the

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time slot for such process because machines are much fast in computational speed than human being and can operate very easily on multiple tasks simultaneously in parallel. In addition the results obtained are more accurate compared to manual effort. In conclusion, even though still advancement needs to be made in this field of study, the result obtained through image processing techniques could be of great benefit to medical sciences.

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