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Phytochemistry Profile and Histopathological Evaluation of *Phaseoleus vulgaris* L beans Ethanolic Extract in Alloxan-Induced Diabetic Rat

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Abstract : *Phaseous vulgaris L* (beans), is one of alternative medicine to treat diabetes mellitus (DM) in Indonesia. We investigated the phytochemistry profiles of ethanol extract of *Phaseous vulgaris* L (EEPV) beans and evaluated the histopathological alterations in diabetic rats. Phytochemical profiles were conducted using Ultraviolet Visible (UV) Spectrophotometer, Infra Red(IR) Spectrophotometer and Gas Chromatography Mass Spectrometry (GCMS). Alloxan (120 mg/kg, intraperitoneally)-induced diabetic rats were divided into 5 group (n=5) i.e. NC: normal, P1: diabetic-control, P2, P3 and P4 (200mg/kg, 400mg/kg and 600mg/kg of EEPV, orally) for 28 days. At the end treatment, the rats were sacrificed to obtain the liver and kidney for histopathological evaluation using Haematoxylin and Eosin technique.UVdata showed the presence of conjugated double bond, whileIR spectra identified some functional groupsi.e. hydroxyl group (OH). GCMS informed us 3 peaks with molecule relatives were (1) 177 (C_{12} $H_{14}O_4$;Molecular Weight(MW):222; Retention Time (RT):5.071), (2) 138 ($C_6H_{10}O$ MW:98 RT: 6.611), (3)147($C_{22}H_{42}O_4$; MW:370 RT:16.148), respectively. The liver and kidney histopathological appearance of P4 showed a complete restoration compared to NC whereas on P1 showed a high destruction.EEPV consist of double bond, hydroxyl, and phenolic functional group and was able to restore the liver and kidney destruction of alloxan-induced diabetic rats at dose 600 mg/kg.

Keywords : Phaseoleus vulgaris L, beans, ethanolic extract, histopathological, diabetic rats.

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Introduction

Diabetes mellitus (DM) is a global public health problem that become emerging as an epidemic world over. According to a widely accepted estimation, the number of diabetic patients would reach 366 million by the year 2030 (Wild *et al*., 2004). Asia and Africa are the two greatest contributor of diabetic population in the world (ADA, 2007).Morbidities and mortalities of diabetic patients are caused by the development of nephropathy, neuropathy, and retinopathy (Atalay, 2002). Liver disease is the most common caused by diabetes which can also cause death(de Marco, 1999). Thus, patients with diabetes have a high prevalence of liver disease and vice versa (Tolman, 2007).

Many herbal medicines have been recommended for the treatment of diabetes(Widyawati *et al*. 2015). The usage of herbs is based on the belief that plant drugs are less toxic and less severe side effects than synthetic ones due to the plethora of active ingredients present in a single herb (Tiwari and Rao, 2002).The amount of active compound content in a herb is also considered as the benefit of its multifactorial action to alleviate DM (Ebong, 2008).*Phaseolus vulgaris L* (PV) have a notable place in the folklore throughout the world and in the traditional usage of many cultures such as antidiabetic (Campillo, 2004; Caraie*t al.*, 2009 and Mishra*et al*., 2010). In Indonesia, this plants commonly grow in Karo lands, especially Berastagi, one of district in North Sumatera, Indonesia. Local residents also often use it to alleviate the rise of blood glucose levels.Previous study showed that oral administration of aqueous extract of *Phaseolus vulgaris L*. (AEPV) (200 mg/kg body weight (BW)) for 45 days to diabetic rats significantly reduced the elevated bloodglucose, serum triglycerides, free fatty acids, phospholipids, total cholesterol, very low density lipoprotein (VLDL) and low density lipoprotein (LDL). The extract also decreased the plasma thiobarbituric acid-reactive substances (TBARS) and hyroperoxides(Venkateswaran, 2002). AEPV at 400 mg/kg bw was also significantly reduce ($p<0.05$) the values of ALT, AST and ALP. Phytochemical screening indicated the presence of alkaloids, flavonoids, saponins, tannins, cyanogenic glycosides, terpenes and steroids (Luka *et al*., 2013).

Currently, the data on antidiabetic properties and bioactive constituent of PV are limited in open literature. Hence, the present study was undertaken to determine the phytochemical profile of ethanolic extract of *Phaseolus vulgaris L*. (EEPV) beans and its histopathological evaluation on liver and kidney in alloxan-induced diabetic rats.

Material and Methods

Chemical and reagents

Alloxan, formalin buffer 10%, heparin sodium, sodium chloride, cell lysis buffer,paraffin wax, TBA reagent, aquabidest, 70% and 80% aqueous alcohol and 96%absolute alcohol, xylol, glyserin, Mayer's haematoxylin, eosin, canada balsem. All other chemicalsused were of analytic grade.

Animals

Wistar rats (*Rattus norvegicus*)was obtained from animal house of UniversitasSumatera Utara. Inclusion criterias are male, 2-3 months old, 150-200 g body weight and haveneverbeen used for other studies.The ratswere maintained on standard pellets and water ad libitum. Permission and approval for animal studies were obtained from the college of Animal Research Ethics Committees (AREEC),Faculty of Mathematicsand Natural Sciences (FMIPA), Universitas Sumatera Utara with number of EC: 115/KEPH-FMIPA/2017.

Induction of diabetes

Alloxan solutionin 0.1 M citric buffer was administered intraperitoneally at single dose of 120 mg/kg BW intraperitoneally. Diabetes was confirmed by determining the blood glucose concentration using glucometer (®glucoDrTM model AGM-2100),after 72 hours injection. The rats with blood glucose level >250mg/dl were selected for the study(Chougale, *et al*., 2007; Nugroho, 2006).

Plant material and preparation of EEPV

The PV beans(Figure.1) were collected at the Berastagi city, Medan (Sumatera Utara) (Figure.2),in February 2017 and wereauthenticated by Department of Botany, Universitas Sumatera Utrara. EEPV was done with maceration method that used ethanol solvent with ratio 1:10 (w/v). The dried PV was dissolved by 10

parts, after that poured with 75 parts of ethanol 96% as the essenced. Closed and left for 5 days and shielded from the light while stirring occasionally. After 5 days the solution was filtered, the pulp was squeezed and washed with enough liquid essence to obtain 100 parts. The extract was obtained evaporated at a temperature of 50^0C.

Figure 1. Phaseolus vulgaris L http://tropical.theferns.info/image.php?id=Phaseolus+vulgaris

Figure 2. Location of Berastagi

Experimental design

The animals were divided randomly into five groups and each group was treated as follows:

Group I (NC) : Normal control rats (standard pellets and water *ad libitum*)for 28 days.
Group II (P1) : Diabetic control rats were administered with 120mg/kg of alloxan, standard pellets and water *ad-libitum* for 28 days.Group III (P2) : Diabetic rats + EEPV at dose of 200mg/kgbw/day for 28 days.
Group IV (P3) : Diabetic rats + EEPV at dose of 400mg/kgbw/day for 28 days.
Group V (P4): Diabetic rats + EEPV at dose of 600mg/kgbw/day for 28 days.

Methods of phytochemistry EEPV

Phytochemistry profile of EEPV was determined by spectrometry methods, namely Ultraviolet Visible (UV) Spectrophotometer,Infra Red (IR) Spectrophotometer, Gas Chromatography Mass Spectrometry (GCMS).

- UV Spectrophotometer Analysis : EEPV was dissolved by *ethyl acetate* solvent and analyzed by Ultraviolet Visible Spectrophotometer Shimadzu UV 1800 at 200-800 nm.
- IR Spectrophotometer Analysis : EEPV was compressed with Kalium Bromide crystal and analyzed by Infra Red Spectrophotometer Shimadzu FT-IR Prestige -21
- GCMS Analysis :1 µl EEPV was injected in GCMS Shimadzu QP 2010- Plus. The GCMS parameters were optimized as follows: injection temperature at 320^{o}C, detector temperature at 320^{o}C and using column temperature programmed started at 100^{o}C and increased gradually until 300^{o}C.

Preparation hepar and kidney for histopathological analysis

At the end of the stipulated 28 days feeds were withdrawn, the rats were subjected to a 12 hours fast but had access to water. Sacrificed using chloroform vapour. Rats were positioned on the surgical board using pins or pin needles. The surgery started in rat stomach by using surgical scissors. The liver and kidney organ were carefully dissected out, trimmed of all fat and connective tissue blotted dry to remove any blood. The tissues were fixed in 10% formal saline, and then transferred to a graded series of ethanol. On day 1, they were placed in 70% alcohol for 7 hours, then transferred to 90% alcohol and left in the latter overnight. On day 2, the tissues were passed through three changes of absolute alcohol for an hour each then cleared in xylene. Once cleared, the tissues were infiltrated in molten paraffin wax in the oven at 58°C. Three changes of molten paraffin wax at one-hour intervals were made, after which the tissues were embedded in wax and blocked out. Prior to embedding, it was ensured that the mounted sections to be cut by the rotary microtome were orientated perpendicularly to the long axis of the hepar and kidney. The sections were designated "vertical sections". Serial sections of 5 µm thick were obtained from a solid block of tissue, fixed on clean albuminized slides to prevent sections coming off the slides and later stained with Haematoxylin and Eosin (H&E) staining techniques, after which they were passed through ascending grade of alcohol, cleared in xylene and mount in DPX mountant, allowed to dry at room temperature and observed histopathologically under digital light microscope.

Photomicrography

Records of the Histopathological results were obtained by photomicrography using digital photomicrographic microscope at the Anatomic Pathology Laboratory, Department of Anatomic Pathology, Universitas Sumatera Utara.

Result and Discussion

Phytochemicalprofile

UV analysis showed the compounds that had conjugated double bond and give absorbance at ultraviolet-visible wavelength 200-800 nm. Spectrum of EEPV was illustrated in Figure.3and Table.1.

Table 1.Absorbance of some peaks at 200-800 nm

No.	P/V	Wavelength	Absorbance
1		662.0	0.05116
2		597.5	0.04419
3		274.0	2.01187

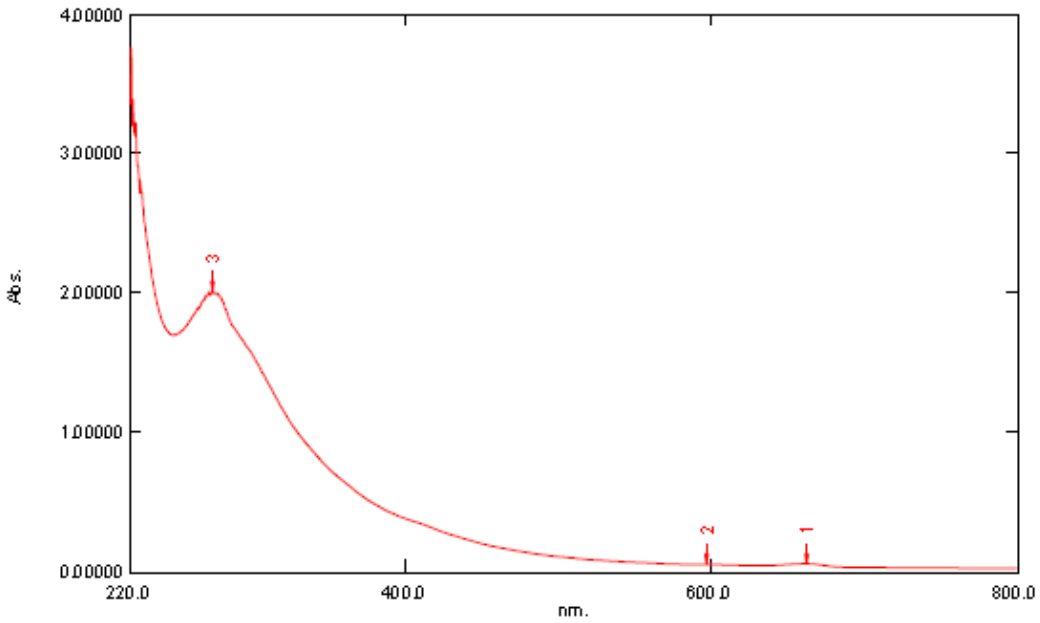


Figure 3. Spectrum of EEPV

The figure depicted that EEPV consist of several compounds. However, only 3 compounds with different wavelength in the spectrum can be seen (Table.1). The maximum absorbance was shown by peak number 3 at 274 nm, followed by peak number 1 at 662 nm and peak number 2 at 597.5 nm. It illustrates the dominant compound in EEPV is number 2 that absorb at ultraviolet wavelength.

IR analysisis used for qualitative analysis, especially functional groups in the compound. The IR spectrum of EEPV is shown in Figure.4.

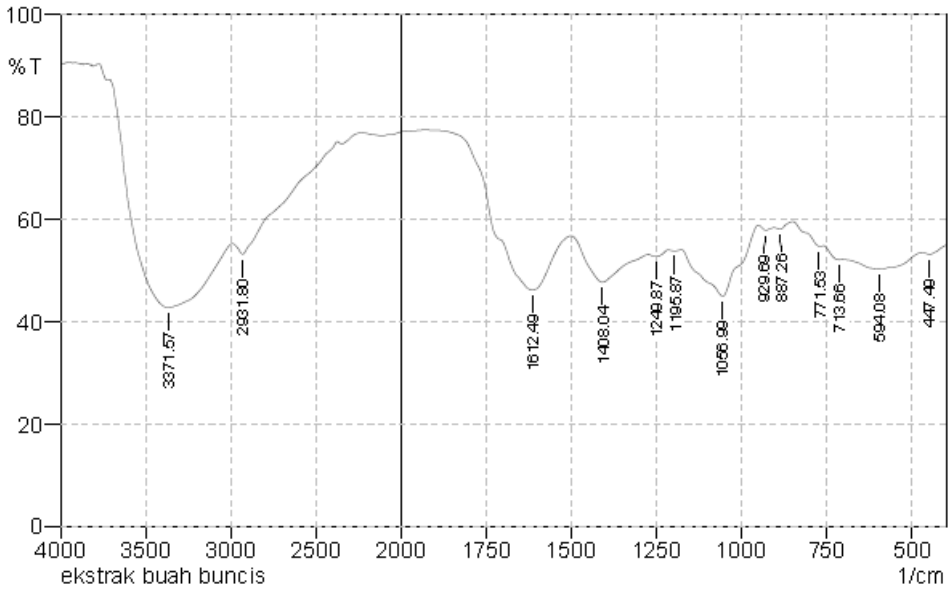
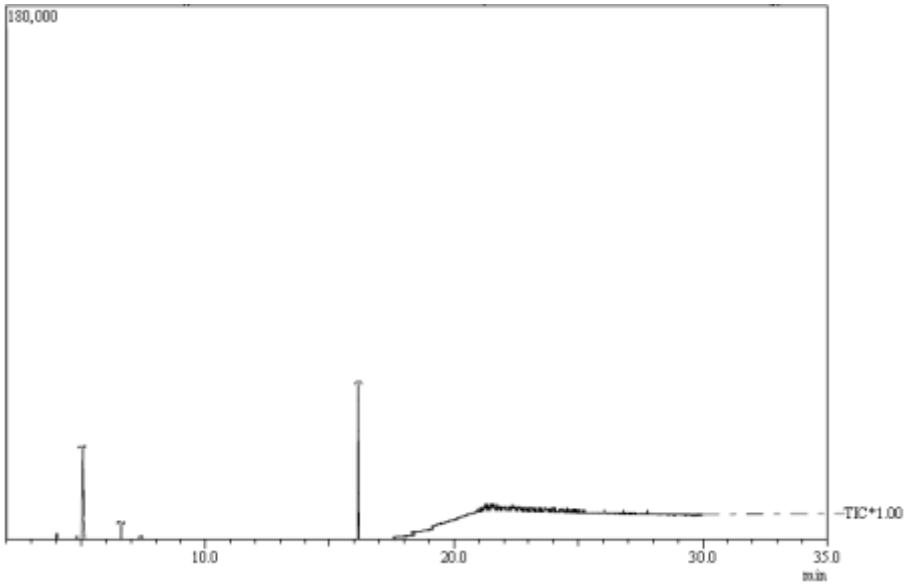


Figure 4. IR Spectrum of EEPV

The spectrum of IR analysis demonstrated that EEPV contained compound with some functional groups. The functional groups were hydroxyl group (OH) that illustrated by broad spectrum peak with wavelength number at 3371,57 cm^{-1}; CH alifatic at 2931,80 cm^{-1} that confimed with another peak at 1408,04 cm^{-1}. Furthermore, the spectrum also informed some peaks in finger print area ensured the functional groups.

GCMS analysisis one of the separation method of organic compounds. The result of GCMS analysis shows the chromatogram profile of EEPV with different molecule relatives and fragmentation models, respectively (Illustrated in Figure.5).



Peak Report TIC

Peak#	R.Time	Area	Area%	Height
1	5.071	86069	46.32	30583
2	6.611	8854	4.77	4988
3	16.148	90886	48.91	52392
		185809	100.00	87963

Figure 5. GCMS Chromatogram of EEPV

In addition, GCMS chromatogram showed some peaks with different percentage area. It informed there was some compounds contained in EEPV with different amount. Moreover, the relative peak area was calculated as following: area of each peak/total area of peaks on chromatogram x 100%. Figure 3 illustrated that EEPV consist of 3 peaks with different Retention Time (RT): (1) 5,071 minutes; (2) 6,611 minutes, (3) 16,148 minutes. Peak number 3 had maximum percentage area compared others by 48,91%. There was asimetric peak in the chromatogram that showed separation of the compounds in extract was not completely.

Furthermore, GCMS analysis informed molecule relatives of each compound with their fragmentation models (Figure.6).

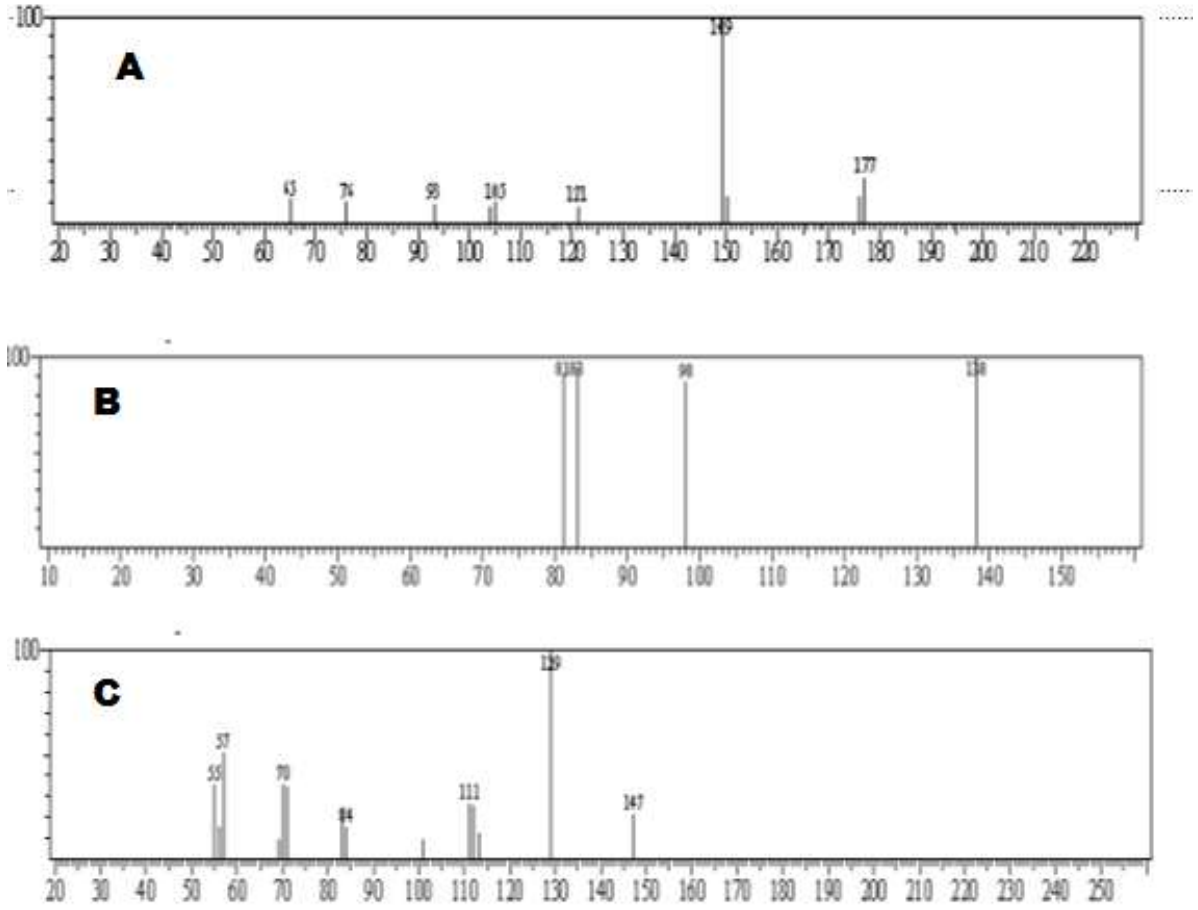


Figure 6. Molecule relatives and Fragmentation models of each peak. Molecule relatives and fragmentation models of first peak (A), second peak (B) and third peak (C).

Table 2. Molecule relatives, retention time, molecular formula, molecular weight and chemical name of each peak GCMS

Peak	Molecule Relative	Retention Time (RT)	Molecular Weight	Molecular Formula	Chemical Name
1	177	5.075	222	$C_{12}H_{14}O_4$	1,2-Benzenedicarboxylic acid, diethyl ester (CAS) Ethyl phthalate $$ Diethyl phthalate $$ Anozol $$ Phthalol $$ Solvanol $$ Neantine $$ Placidol
2	138	6.608	98	$C_6H_{10}O$	2,4-Hexadien-1-ol (CAS) 2,4-Hexadiene-1-ol $$ Hexacose $$ Hexakose $$ 2,4-Hexadienol $$ Sorbyl alcohol $$ Sorbic alcohol $$ Sorbinic alcohol
3	147	16.150	370	$C_{22}H_{42}O_4$	Di(2-ethylhexyl)adipate $$

Table.2 demonstrated each peak as a result of GCMS had different molecule relatives, molecular formula, molecular weight and chemical name. Alloxan is a very unstable chemical compound with a molecular shape resembling glucose (Lenzen and Munday, 1991). Both alloxan and glucose are hydrophilic and do not penetrate the lipid bilayer of the plasma membrane. The alloxan molecule is structurally so similar to glucose that the GLUT2 glucose transporter in the beta cell plasma membrane accepts this glucomimetic and transports it into the cytosol. Alloxan does not inhibit the function of the transporter, and can therefore selectively enter

beta cells in an unrestricted manner (Elsner, 2002). In the presence of intracellular thiols, especially glutathione, alloxan generates reactive oxygen species (ROS) in a cyclic redox reaction with its reduction product, dialuric acid. Autoxidation of dialuric acid generates superoxide radicals, hydrogen peroxide and in a final iron-catalysed reaction step, hydroxyl radicals. These hydroxyl radicals are ultimately responsible for the death of the beta cells, which have a particularly low antioxidative defence capacity, and the ensuing state of insulin-dependent 'alloxan diabetes' (Lenzen, 2008).

In this study, the spectrometric methods showed the phytochemical profile of EEPV containing 3 compounds had conjugated doublebond (Figure.6). The conjugated double bond can provide electrons thereby preventing oxidative stress, which may be involved in severe damage of cell molecules and structures. Oxidative stress is produced during normal metabolic process in the body as well as induced by a variety of environmental factors and chemicals. Oxidative stress has been shown to have a significant effect in the causation of diabetes as well as diabetes-related complications in human beings(Wilson, 1998; Widyawati, 2016). Oxidative stress in diabetes has been shown to coexist with a reduction in the antioxidant status. The exact role of oxidative stress in the etiology of human diabetes is however not known. Oxidative stress has been shown to produce glycation of proteins, inactivation of enzymes, and alterations in structural functions of collagen basement membrane (Baynes, 1991). Oxidative stress may have significant effect in the glucose transport protein (GLUT) or at insulin receptor. Scavengers of oxidative stress may have an effect in reducing the increased serum glucose level in diabetes and may alleviate the diabetes as well as reduce its secondary complications(Abolfathi *et al.,* 2012)

Histopathological evaluation

Histopathological evaluation of liver and kidney on alloxan-induced diabetic rats was illustrated in Tabel.3, Figure.7 and Figure.8.

Table 3. Histopathological evaluation of liver and kidney on alloxan-induced diabetic rats treated with EEPV

Group	Histopathological Evaluation	
	Liver	**Kidney**
NC: Normal Control	normal cellular architecture	normal cellular architecture
P1 : Diabetic Control	irregular hepatocytes, increase in sinusoidal space (SS) dilation and glycogen accumulation	vascular congestion, tubular necrosis,glomerular inflammation, epithelial membran lining degeneration
P2 : Diabetic+EEPV 200mg/kgbw	sinusoidal space (SS) dilation and glycogen accumulation	vascular congestion, focal tubular and glomerular inflammation
P3 : Diabetic+EEPV 400mg/kgbw	Reduced degree of sinusoidal and central vein dilations	Reduced vasular congestion and glomerular inflamation
P4 : Diabetic+EEPV 600mg/kgbw	complete restoration	Increased celluler regeneration

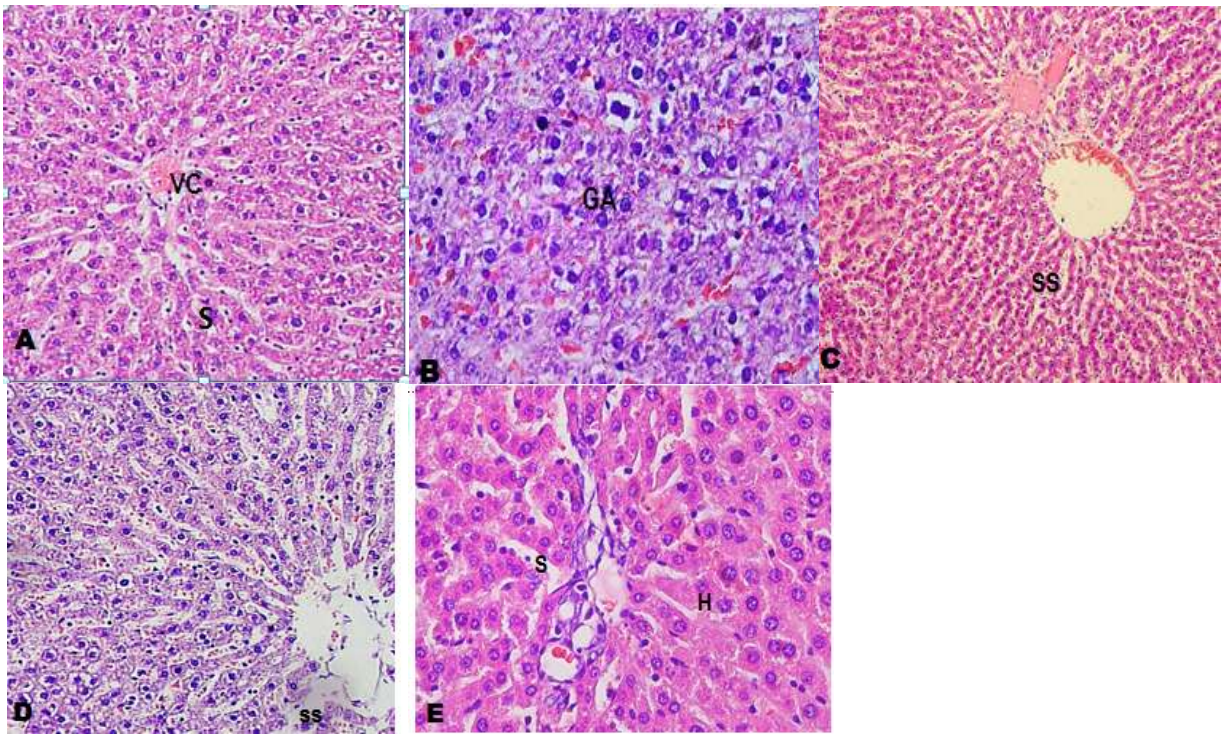


Figure 7. The photomicrographs (10×10) of liver section. NC group (A) showed a normal structure of central vein surrounded by hepatic cells; (B). DC group showedirregular hepatocytes and glycogen accumulation; (C). DC received EEPV (200 mg/kgbw) showed sinusoidal space (SS) dilation and glycogen accumulation; (D). DC received EEP (400 mg/kgbw) showed reduced sinusoidal space (SS) dilation; (E). DC received EEP (600mg/kgbw) showed cellular regeneration similar to NC (H&E).

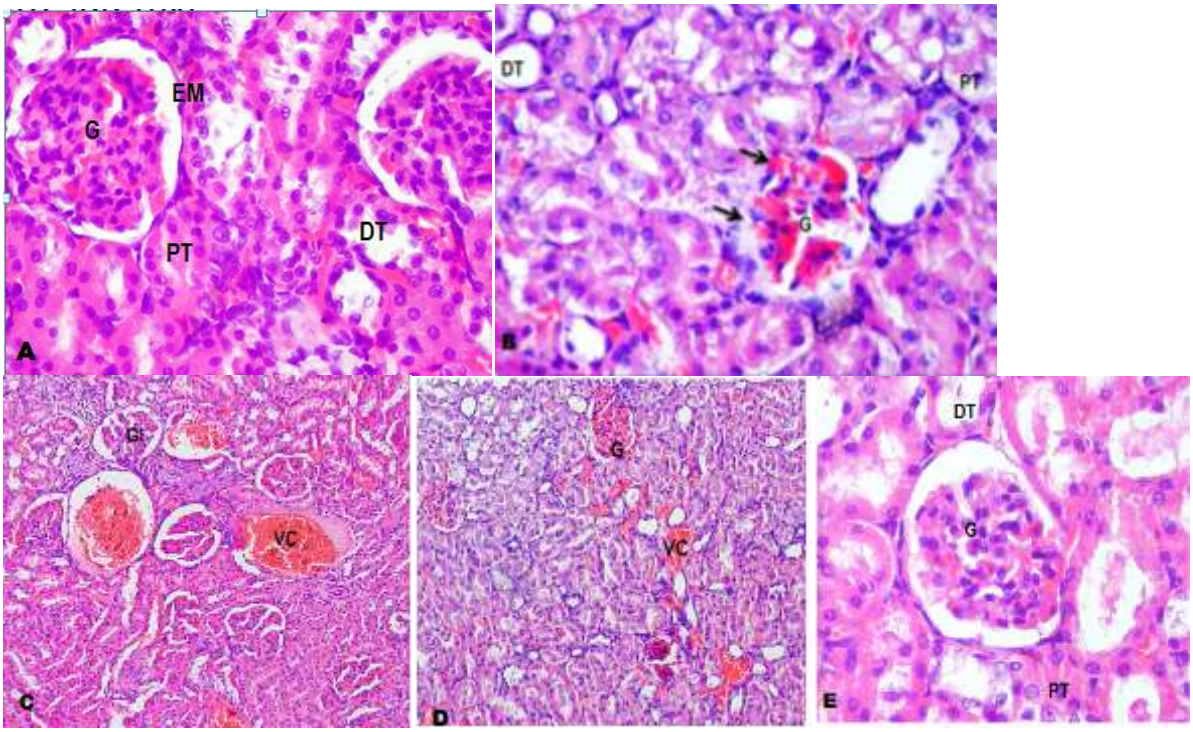


Figure 8. The photomicrographs (40×10) of kidney section. NC group (A) showed a normal structure of glomerulus (G) and tubulus (T); (B). DC group showedvascular congestion, tubular necrosis, glomerular inflammation, epithelial membran lining degeneration (arrow), oedem tubulus; (C). DC received EEPV (200 mg/kgbw) showedvascular congestion (VC), focal tubular and glomerular inflammation (GI);(D). DC received EEP (400 mg/kgbw) showed reduced vasular congestion and glomerular inflamation; (E).DC received EEP (600mg/kgbw) showed increased cellular regeneration similar to NC (H&E).

The histopathological evaluation of this study also revealed that daily treatment of EEPV for 28 days markedly improve tissue repairment of liver tissue of alloxan-induced diabetic rats. This improvement was suggessted by the ability of EEPV to provide antioxidant activity. The liver tisssue assesment of this study result supported a reversible effect evidence, from mild to complete restoration. NC group were found to be stable, whereasP1 showed high level of cellular abnormalities, including irregular hepatocytes, sinusoidal space dilation and glycogen accumulation (Figure .7). As well as kidney showed increased celluler regeneration after treated with EEPV at dose 600mg/kgbw (Figure.8).

Phytochemical screening of PV indicated the presence of alkaloids, saponins, tannins, cyanogenic glycosides, terpenes, steroids and flavonoids (Luka *et al.,* 2013).Flavonoids were known as compounds that have antioxidant activity due to its ability to capture the free radicals in the body that called as radical scavenger. Their actions were yielded by the conjugated bond and Hatom as the donor of the hydroxyl (-OH) phenolic. This natural antioxidant can prevent the damaging caused by free radicals, improve the function of organs. such as pancreatic organ (Patel *et al.,* 2010). By repairing the function of the pancreas, the high of blood glucose levels can be reduced, thus the damaging of liver and kidney can be improved. The present of the conjugated bond and OH-function in EEPV suggested their contribution to its activity to repair liver and kidney destruction in alloxan-induced diabetic rats.

Conclusion

Phytocemical profile of EEPV identify 3 compounds that consist of double bond and H atom. These compounds are known as the donor of the hydroxyl (-OH) phenolic that act as free radical scavengers. EEPV at dose 600mg/kgbwable to restore the liver and kidney destruction in alloxan-induced diabetic rats.

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Conflicts of Interest

The author declare no conflicts of interest.

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