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The protective effect of *Hypericum organifolium* in experimental renal ischemia/reperfusion injury in rats

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***Hypericum organifolium* leaf extract (HOE) is a medicinal plant extract containing many polyphenolic compounds. Polyphenolic compounds have high antioxidant potential. Reactive oxygen metabolites (ROMs) play a role in the pathogenesis of ischemia/reperfusion injury (I/R) in the kidney. This study was designed to determine the possible protective effect of HOE on renal I/R injury. Twenty four adult female Wistar albino rats were evaluated in three groups. Group 1 (Control), group 2 (renal I/R injury+Saline), and group 3 (renal I/R injury+HOE 50 mg/kg) were designed to evaluate the effects of HOE in renal I/R injury on histopathological examinations. The malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase (CAT) activities were determined. Plasma blood urea nitrogen (BUN), creatinine (Cr), uric acid (UAC), SGOT (alanine aminotransferase, ALT), SGPT (serum aspartat aminotransferase, AST) and lactate dehydrogenase (LDH) levels were measured. HOE treatments in renal ischemia-reperfusion decreased MDA in kidney and liver. SOD and CAT activities were increased with HOE treatment in kidney and liver. When all groups were compared histopathologically in kidney, HOE administration improved I/R-induced damages such as hyaline cast, tubular dilatation and parenchymal hemorrhagia. The plasma BUN values were increased in I/R group when compared with control; on the other hand after HOE administration, the BUN values decreased, but not significantly. The Cr, UAC, SGOT, SGPT and LDH levels increased in I/R group, but decreased after HOE administration when compared with I/R group. The findings imply that ROMs play a causal role in I/R-induced renal injury and HOE exerts renoprotective effects probably by the radical scavenging and antioxidant activities**

Key words: *Hypericum organifolium*, renal ischemia/reperfusion, rat, antioxidant.

INTRODUCTION

Acute renal failure (ARF) resulting from renal ischemia reperfusion injury (IRI) remains a major clinical problem

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encountered in many clinical situations: kidney transplantation, partial nephrectomy, renal artery angioplasty, cardiopulmonary bypass, aortic bypass surgery, accidental or iatrogenic trauma, sepsis, hydronephrosis, and elective urological operations. Although, the return of blood flow to ischemic tissue can result in the recovery of normal function, paradoxically the tissue may also be injured during the process of reperfusion (Sehirli et al., 2008; Yun et al., 2009). Renal ischemia initiates a complex and interrelated sequence of events, resulting in the injury and death of renal cells. Although, reperfusion is essential for the survival of ischemic renal tissue, it causes additional damage contributing to the renal dysfunction and injury associated with I/R of the kidney (Thandani et al., 1996; Lien et al., 2003). Especially, the cells of the proximal tubular epithelial are susceptible to I/R injury, leading to acute tubular necrosis, which plays a pivotal part in the pathogenesis of ARF (Lieberthal and Levine, 1996; Avlan et al., 2006). Reperfusion of the ischemic tissue produces reactive oxygen species, which are known to have deleterious effects such as increased microvascular permeability, interstitial edema, impaired vasoregulation, inflammatory cell infiltration and necrosis. Oxidative stress is a relative excess of oxidants caused by increased free radical production and/or decreased antioxidant defense systems that impairs cellular function and contributes to the pathophysiology of many diseases (Zhao, 2005; Karimi et al., 2005). The antioxidant defense systems, non enzymatic free radical scavengers (e.g., vitamin E, vitamin C, uric acid, and bilirubin) and the antioxidant scavenging enzymes, (catalase [CAT], superoxide dismutase [SOD], and glutathione peroxidase [GPx]) protect cells and tissues against oxidative injury (Marubayashi and Dohi, 1996; Zhao, 2005). The genus *Hypericum* L., a member of the Guttiferae (Hypericaceae) family, contains about 400 species in the world. In Turkey, the genus is represented by about 80 species (Robson, 1967, 1988). It has traditionally been used on a widespread basis almost all over the world. *Hypericum origanifolium* extract (HOE) include naphthodiantrones (hypericin, pseudohypericin, proto-pseudohypericin, emodin, and frangulin), flavonoids (quercetin, myricetin, and hyperoside), and xanthenes (mangiferin and isomangiferin) (Mathis and Ourisson, 1964; Makovestška, 1999; Sirvent et al., 2002; Kitanov and Nedialkov, 1998). The polyphenolic compounds such as *Hypericum perforatum* have high antioxidant and preventive effect against damages of ischemia/reperfusion (I/R) injury (Abolfathi et al., 2011). There is increasing evidence to suggest that reactive oxygen metabolites (ROMs) play a role in the pathogenesis of I/R in the kidney.

We designed this study to determine the possible protective effect of HOE against oxidative stress during I/R injury of the kidney in rat via renal biochemical and histological and hepatic biochemical parameters.

MATERIALS AND METHODS

The experimental protocols were approved by the institutional animal ethics committee. Animals were obtained from the medical and surgical experimental research center of the institute and all experiments were carried out in the same center.

Animals

Twenty four adult female Wistar albino rats weighting 220 to 250 g were evaluated in three groups. All rats were housed in polycarbonate cages in a room with controlled temperature ($22 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$), and a 12 h cycle of light and dark and were fed laboratory pellet chows and water was given *ad libitum*. The experiment was performed after a stabilization period in the laboratory for several days. All the rats used in the following experiments were subject to the Guiding Principles for the Care and Use of Laboratory Animals and the Recommendations of the Declaration of Helsinki.

Experimental design

Group 1 (Control), group 2 (renal I/R injury + saline), and group 3 (renal I/R injury + 50 mg/kg HOE) were designed to evaluate effects of HOE in renal I/R injury on the morphological changes (Öztürk, et al., 2009). Right nephrectomy was performed before 15 day renal I/R, except for group 1. HOE was administered (50 mg/kg, interperitoneally (i.p.)) 15 min prior to ischemia. Renal I/R injury was induced with left renal pedicle occlusion for 45 min, followed with reperfusion for 6 h under anesthesia. After induction of I/R injury, left nephrectomies were performed for histopathological examinations.

Surgical procedures and HOE administration

Drug administration

Under xylazine (10 mg/kg) and ketamine (70 mg/kg) anesthesia, a right nephrectomy was performed and the rats were allowed to recover for 15 days before they were subjected to I/R injury. On the 15th day following nephrectomy, rats were fasted overnight. The renal pedicle was occluded for 45 min to induce ischemia and then subjected for 24 h of reperfusion (I/R groups). HOE was dissolved in serum physiologic as 2 ml/kg and was given as 50 mg/kg; I/R+HOE groups. HOE or serum physiologic (I/R group) was administered intraperitoneally 15 min before ischemia and 12 h after reperfusion. The animals were decapitated after 24 h of reperfusion period. After induction of I/R injury, left kidney and liver were processed for histopathological examinations

Histopathological evaluation

Left nephrectomy specimens and livers were processed routinely in 10% formalin solution, and embedded in paraffin. Tissue sections of 5 μm were obtained, and stained with hematoxylin and eosin (H and E). Histopathological examinations were performed under a light microscope (NIKON, Japan). A minimum of 10 fields for each kidney slides with minimum X50 magnification were examined to assign the severity of these morphological changes. The morphological changes were scored on a scale of none (-), mild (+), moderate (++) , and severe (+++) damage in order to perform a

comparison between the groups.

Biochemical analysis

Blood samples were collected to determine blood urea nitrogen (BUN), creatinine (Cr) and uric acid (UAC) as indicators of kidney function, alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) to assess liver function. In addition, lactate dehydrogenase (LDH) was assayed in serum samples for the evaluation of generalized tissue damage. These were determined spectrophotometrically using an automated analyzer.

Post mitochondrial supernatant preparation (PMS)

After sacrificing the animals, isolated areas of the nephron of their kidneys were quickly removed and perfused immediately with ice-cold normal saline, and homogenized in chilled potassium chloride (1.17%) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 800 xg for 5 min at 4°C in a refrigerated centrifuge to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 xg for 20 min at 4°C to get the PMS which was used to assay malondialdehyde (MDA), catalase (CAT), and SOD activity.

Protocols of lipid peroxidation and enzyme activities measurement

MDA production is an end product of lipid peroxidation which reacts with thiobarbituric acid to form a red colored complex. The measurement of MDA levels by thiobarbituric acid reactivity is the most widely used method for assessing lipid peroxidation. 0.1 ml of homogenate, 3 ml of 1% phosphoric acid, and 0.5 ml of distilled water and 1.0 ml of 0.6% 2-thiobarbituric acid were added. The mixture was boiled in water bath for 45 min. Afterward, the mixture was cooled in on ice, followed by an addition of 4.0 ml of n-butanol to extract the cold thiobarbituric acid reactants. The optical density of the n-butanol layer was determined at 532 nm after centrifugation at 1,000 g for 5 min and was expressed as nmol MDA/g of tissue (Mihara and Uchiyama, 1978).

Determination of SOD activity

SOD activity was spectrophotometrically assayed with commercial kits. The Fluka SOD kit USA contains all reagents and solutions required for determining SOD activity in an indirect assay method based on xanthine oxidase and a novel color reagent. The homogenate SOD activity was determined by inhibition of Formosan dye (450 nm) employing the xanthin-xanthin oxidase enzymatic method to generate superoxide radicals and expressed as U/g.

Determination of CAT activity

One unit of CAT equals the enzyme activity that recognized 1 µmol of hydrogen peroxide in 60 s at 37°C. The three blank samples were prepared according to Goth (1991). CAT activity was measured with determination of absorbance of three blank samples at 405 nm in spectrophotometer. CAT activity (kU/L) was calculated as = $[(Abs_{blank1} - Abs_{blanksample}) / (Abs_{blank2} - Abs_{blank3})] \times 271$ (Goth, 1991).

Statistical analysis

The statistical analysis was performed with the computer program "Statistical Package for Social Sciences for Windows" (SPSS Inc; Release 11.5; Sep 6, 2002). All of the data were expressed as means ± standard deviation (SD). Differences between groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. The significance was tested at non significant (ns) $p > 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

The control group did not show any morphological changes. By contrast, hyaline cast was present in all the rats in group 2 (severe in 1, moderate in 4 rats, AND mild in 3 rats). Tubular dilation and parenchymal hemorrhagia were detected in 7 rats (moderate in 4 rats and mild in 3 rats) and in 5 rats (moderate in 3 rats and mild in 2 rats) in group 2, respectively. In group 3 (renal I/R + 50 mg/kg HOE), mild in 1 parenchymal hemorrhagia and mild hyaline cast were present in 4 rats, and there were normal histopathological findings in 3 rats (Figures 1 and 2). We showed that MDA levels of group 2 (I/R) in renal tissue were significantly higher than control group. HOE administration had significantly decreased MDA levels in groups 3 when compared with group 2. When we compared the SOD levels, it significantly decreased in group 2 when compared with control group. But the increase of SOD levels was statistically significant in group 3 when compared with group 2. The CAT levels decreased significantly in group 2 when compared with control group, but after HOE administration CAT levels increased. The enzymatic activity changes in kidney are presented in Table 1. MDA levels of group 2 (I/R) in liver were significantly higher than control group, but it was lower in group 3 than in group 2. When we compared the SOD levels, it significantly decreased in group 2 when compared with the control group. The SOD levels in group 3 increased significantly when compared with group 2. The CAT levels decreased significantly in group 2 when compared with control group, but after HOE administration, CAT levels increased in group 3.

These results suggested that the other organs such as liver may be affected in renal I/R injury. HOE may be effective in preventing oxidative injury for other organs and tissues. The enzymatic activity changes in liver are demonstrated in Table 1. The plasma BUN values increased in I/R group when compared with the control; on the other hand after HO administration, the decrease of the BUN values were not significant. The Cr level increased in I/R group, but decreased after HO administration when compared with group 2. The UAC level were increased in I/R group, but decreased after HO administration when compared with group 2. The ALT, AST, and LDH levels increased in I/R group, but decreased after HO administration when compared with

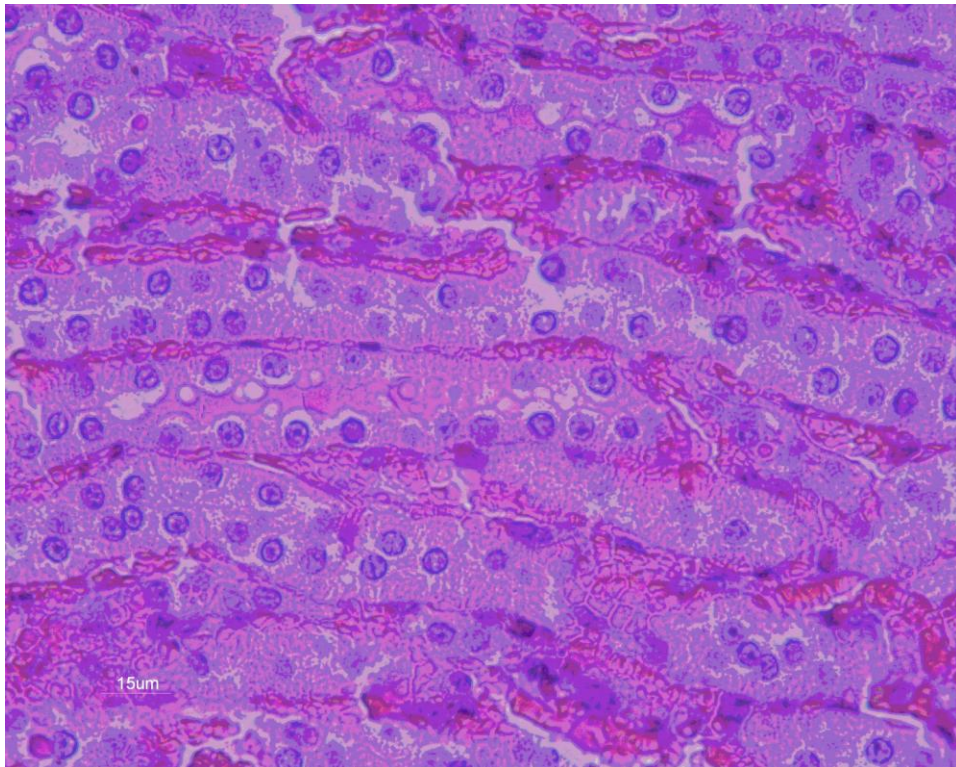


Figure 1. Parenchymal hemorrhage (++) H&E, Scale bar: 15 μ m.

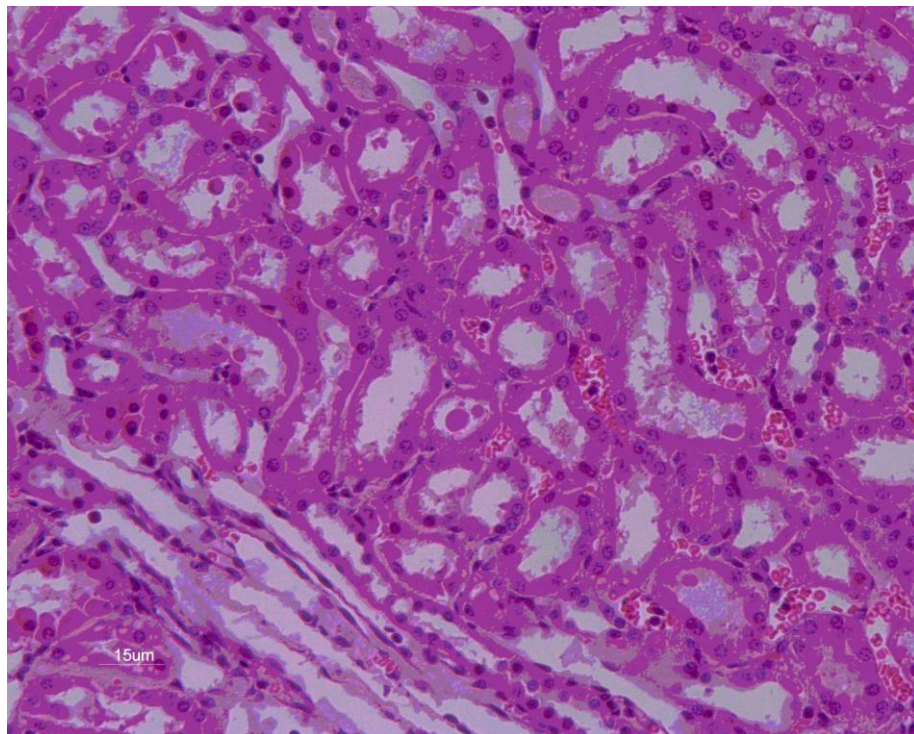


Figure 2. Hyaline cast (+) and tubular dilatation (++) H&E, Scale bar: 15 μ m.

Table 1. MDA, SOD, and CAT levels in kidney and liver tissue after I/R.

Group	MDA		SOD		CAT	
	Kidney	Liver	Kidney	Liver	Kidney	Liver
Group 1	5.31 ± 0.59	4.81 ± 0.14	18.01 ± 0.96	38.47 ± 8.39	571.71 ± 38.64	478.64 ± 112.73
Group 2	10.44 ± 1.20	4.15 ± 0.53	12.32 ± 1.09	11.63 ± 4.07	420.71 ± 41.51	112.77 ± 5.16
Group 3	6.97 ± 0.72	5.38 ± 0.31	16.07 ± 0.65	39.30 ± 9.39	457.85 ± 38.67	192.00 ± 94.61
<i>P</i> -values and multiple comparison of the groups	G1 - G2.***	G1 - G3 ^{ns}	G1 - G2***	G1 - G3 ^{ns}	G1 - G2***	G1 - G3***
	G1 - G3.**	G1 - G2*	G1 - G3**	G1 - G2**	G1 - G3***	G1 - G2***
	G2 - G3.***	G3 - G2***	G2 - G3***	G3 - G2***	G2 - G3. ^{ns}	G3 - G2 ^{ns}

^{ns}*p* > 0.05. **p* < 0.05; ***p* < 0.01; and ****p* < 0.001.

Table 2. The means and standard deviations (±SD) for biochemical analysis of groups.

Group	BUN (mg/dl)	CRE (mg/dl)	UAC (mg/dl)	ALT (U/L)	AST (U/L)	LDH (U/L)
Control	38.65 ± 2.11	0.52 ± 0.056	1.22 ± 0.08	78.10 ± 2.53	66.61 ± 7.29	120.21 ± 13.11
I/R	108.22 ± 16.70	1.50 ± 0.22	2.80 ± 0.22	264.82 ± 9.45	94.61 ± 4.68	658.65 ± 47.79
HOE	98.37 ± 6.33	1.06 ± 0.29	2.36 ± 3.28	158.71 ± 11.68	73.98 ± 2.61	430.70 ± 25.90

group 2 (Table 2).

DISCUSSION

The acute renal failure produced by I/R is a clinical and experimental syndrome characterized by major reduction in glomerular filtration rate, extensive tubular damage, tubular cell necrosis, glomerular injury, and signs of tubular obstruction with cellular debris (Kabasakal et al., 2005). Renal I/R induced acute tubular necrosis is observed most frequently in patients after cardiac and aortic operations, trauma, severe dehydration, burns, and others. Because renal failure induced by these conditions is a devastating problem, evaluation of new therapeutic agents is essential (Avlan et al., 2006). Thus, to assess its protective effect in renal I/R injury, we investigated HOE. In our study, HOE treatments in renal ischemia-reperfusion decreased MDA in kidney and liver. SOD and CAT activities were increased with HOE treatment in kidney and liver. When all groups were compared histopathologically in kidney, HOE administration improved I/R-induced damages such as hyaline cast, tubular dilatation, and parenchymal hemorrhagia. The plasma BUN values increased in I/R group when compared with the control group; on the other hand after HOE administration, the BUN values decreased, but not significantly. The Cr, UAC, SGOT, SGPT, and LDH levels increased in I/R group, but decreased after HOE administration when compared with I/R group. Takahira et al.

(2001) showed that treatment with dexamethasone did not ameliorate serum creatinine concentration, although dexamethasone attenuated enhanced neutrophil infiltration induced by renal I/R. Oztürk et al. (2001) showed that there are significant improvements in serum BUN and creatinine concentrations in the molsidomine treated and L-N^G-nitroarginine methyl ester (L-NAME) treated animals. In addition, molsidomine improved renal damage; although, L-NAME did not improve as compared to I/R group. According to Takaoka et al. (2002), treatment with low dose Lipoic Acid (LA) tended to attenuate the histological damage, but its effects were not significant. The higher dose of LA significantly attenuated the development of all these lesions and suppressed BUN and creatinine elevation (Takaoka et al., 2002). Uz et al. (2009) found that the levels of urea, creatinine levels remained unchanged in ginger + I/R group as compared to I/R group. SOD enzyme activity was significantly increased by the treatment with ginger, but CAT activity and levels of MDA did not change. Histological examination of the kidneys subjected to I/R process showed the distinctive pattern of ischemic renal injury, which included widespread degeneration of tubular architecture, loss of brush border, sloughing tubular epithelial cells from the basement membrane, tubular cell necrosis, and intratubular cast formation, especially in the outer medulla. Ginger + I/R group demonstrated marked reduction of the histological features of renal injury (Uz et al., 2009). Yanarates et al. (2008) observed minimal tubular cell swelling, brush border loss, and nuclear

condensation in kidney section of plasminogen activator (PA) treated rat as compared to I/R rat. PA significantly reduced the I/R-induced increases in Cr, BUN, and AST. In addition, PA restored decreased antioxidant enzymes, and attenuated histological alterations. They emphasized that PA may serve as a potential therapeutic agent in protecting kidney and multiple target organs from I/R injury (Yanarates et al., 2008). Rhoden et al. (2001) used different times of reperfusion after the renal ischemia. The data revealed that the renal ischemia had significantly increased serum creatinine levels at 24 and 96 h after the surgical procedure, when compared with the control group. But it did not change at 192 h. Furthermore, alfa-tocopherol significantly protected renal function in rats subjected to renal ischemia and reduced the MDA concentration. Dietary deficiency of vitamin E seems to lead to greater structural and functional renal impairment and increased lipid peroxidation following renal ischemia (Rhoden et al., 2001). Sucu et al. (2002) investigated the effects of trimetazidine (TMZ) on tissue damage in kidney after hindlimb ischemia/reperfusion (I/R), to evaluate the distant organs after I/R. They observed that, there was a prominent tubulointerstitial injury with loss of prominent brush border. Although, there is no statistical significant difference, loss of Bowman's space, increase in glomerular congestion, and bleeding in periglomerular and peritubular areas were observed in group I/R rather than TMZ-treated group. But leukocytic infiltration was decreased in TMZ group (Sucu et al., 2002). Unlü et al. (2003) observed decreased MDA levels and increased SOD activities in Daflon group. The histopathological evaluation showed that there was a prominent tubulointerstitial injury with loss of brush border and this degeneration was accompanied by segmental glomerular degeneration also for both control group and group D. But the leukocytic infiltration decreased in Daflon group (Unlü et al., 2003). Foglieni et al. (2006) showed that ethylenediaminetetraacetic acid (EDTA) preserved the architecture of kidneys submitted to I/R and improved necrosis, vacuolization, dilation and focal cast in tubular structures, glomerular hemorrhage in kidney after renal I/R. Kabasakal et al. (2005) showed decreased BUN, Cr, and MDA levels with aqueous garlic extract (AGE). Furthermore, AGE improved damage at microscopic level (Kabasakal et al., 2005). Avlan et al. (2006) demonstrated significant reduction in MDA levels in I/R+trapidil group. In addition, serum urea and AST levels were decreased with trapidil treatment. I/R group showed significantly higher histopathological injury scores when compared with scores of other groups (Avlan et al., 2006). As seen, there are a lot of studies which examine the effects of the different antioxidants on renal I/R injury. They revealed different results for enzymatic activities and severe histopathological findings. Our results are in concordance with the result seen

in some of them, while different from some of them. The findings of our study indicate that HOE may have protective role in I/R damage of organs such as kidney and liver by restoring antioxidants and then reducing lipid peroxidation in renal and hepatic tissue.

Conclusions

The findings imply that ROMs play a causal role in I/R-induced renal injury and HOE exerts renoprotective effects probably by the radical scavenging and antioxidant activities.

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Full Length Research Paper

***In vitro* protective effect of *Schisandra chinensis* extract against carbon tetrachloride–induced hepatotoxicity in common carp (*Cyprinus carpio*)**

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In the present study, *in vitro* hepatoprotective effect of *Schisandra chinensis* extract (SCE) was evaluated against carbon tetrachloride (CCl₄)–induced hepatotoxicity in common carp. SCE (100, 200, and 400 µg ml⁻¹) was added to the carp primary hepatocytes before (pre-treatment), after (post-treatment), and both before and after (pre and post-treatment) the exposure of the hepatocytes to 8 mM CCl₄ in the culture medium. Results showed that exposure of the primary cultured carp hepatocytes to 8 mM CCl₄ for 4 h caused cytotoxicity, manifested by loss of cell viability and significantly elevated levels of lactate dehydrogenase (LDH), glutamate oxalate transaminase (GOT), glutamate pyruvate transaminase (GPT) and malondialdehyde (MDA), and significantly reduced activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the supernatant. Pre-treatment and pre and post-treatment of the hepatocytes with SCE significantly reduced the elevated levels of LDH, GOT, GPT and MDA; increased the reduced activities of SOD and GSH-Px and increased the cell viability in a dose-dependent manner. Post-treatment of the hepatocytes with SCE did not show significant effects on the tested parameters except GPT. The results suggest that SCE is a potent hepatoprotective agent that could protect fish hepatocytes against the acute injury and this ability might be attributed to its antioxidant potential. The results also imply that SCE can be potentially used for preventing rather than curing liver diseases in fish.

Key words: Carp primary hepatocytes, hepatoprotection, antioxidant, *Schisandra chinensis*.

INTRODUCTION

In aquatic environment, fish are directly exposed to various natural and synthetic chemicals originated from agricultural and industrial activities. Liver is prone to xenobiotic-induced injury because of its central role in xenobiotics metabolism, its portal location within the circulation, and its anatomic and physiologic structure (Sturgill and Lambert, 1997). Fish liver neoplasm due to chemical challenge has been frequently reported in

polluted areas (Malins et al., 1988; Myers et al., 1991; Myers et al., 2003; Stehr et al., 1997; Koehler, 2004). The use of chemicals in aquaculture systems for various purposes is widely recognized, especially in intensive pond aquaculture system in Asia. The heavy use of prophylactic antibiotics in aquaculture has become a growing problem for human and animal health and for the environment (Cabello, 2006). An increasing number of

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chemicals have shown the potential to induce lesions in the liver of fish (Dixon et al., 1987; Webb et al., 2008; Braunbeck et al., 1990; Oulmi et al., 1995), antimicrobial agents are a common and important cause of hepatotoxicity (Thiim and Friedman, 2003). Oxytetracycline-induced liver injury has been reported in rainbow trout and Atlantic salmon (Brown and Desmond, 2002; Thiim and Friedman, 2003).

Recently, fish disease called hepatobiliary syndrome, with the symptoms of liver and gall bladder enlargement (up to 2 to 3 times of their original sizes) and colour changing, has been frequently reported in many cultured species and caused dramatic loss in China. Histological and biomedical investigations revealed the hepatocyte necrosis and increases in activities of serum glutamate oxalate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in grass carp suffering from hepatobiliary syndrome (Liu et al., 2009). It is a non-infectious disease, pathogenic bacteria or viruses have not been isolated, and it was proposed that xenobiotic challenge due to drug abuse may be one of the important causes of the disease (Shi and Wei, 2010). So far, no effective methods have been found for the treatment of hepatobiliary syndrome, and much attention has been focused on the use of Chinese medicinal herbs to prevent and control this disease (Li et al., 2011).

CCl₄-induced hepatocyte damage is the best-characterized system of the xenobiotic-induced hepatotoxicity, it is frequently used to screen hepatoprotective agents including nutritional supplements and liver protective drugs (Rechnagel and Glende Jr, 1973), and it is also widely used for the study of hepatoprotective effects of drugs and herbal extracts in mammals (Ahsan et al., 2009). However, most of our understanding of CCl₄-induced hepatotoxicity remains confined to mammal models (Guillouzo, 1998), and data obtained in mammal cannot be extrapolated with certainty to the fish situation. Therefore, to screen the hepatoprotective Chinese medicinal herbs specific for liver disorder in fish, an *in vitro* model of CCl₄-induced hepatotoxicity in primary cultured carp hepatocytes was previously established in our laboratory, and it has been successfully used to evaluate the hepatoprotective and antioxidant effects of *Glycyrrhiza glabra* extract in fish (Yin et al., 2011). Primary cultured hepatocytes generally maintain many of their original differentiated *in vivo* characteristics and therefore facilitates extrapolation of the results to the *in vivo* situation (Pesonen and Andersson, 1997).

Schisandra chinensis is a traditional Chinese herb clinically prescribed for the treatment of various liver diseases in human beings because of its capability to protect the liver from injuries induced by various hepatotoxins (Zhu et al., 1999). Lignans including schizandrin A, B and C, schizandrol A and B, schizandrer A and B have been identified from the extract of *S. chinensis* and proven to have hepatoprotective effects against hepatic dysfunction induced by various chemical hepatotoxins in mammals (Xie et al., 2010). In fish, however, the hepatoprotective

effect of *S. chinensis* has not been studied and its corresponding mechanisms have not been demonstrated yet. The present study is aimed at studying the effects of *S. chinensis* extract (SCE) on the function of fish hepatocytes using an *in vitro* model of CCl₄-induced hepatocyte injury and finding out whether it can be potentially used as a medicine for fish hepatobiliary syndrome.

MATERIALS AND METHODS

Chemicals

L-15 medium, ethylenediaminetetraacetic acid (EDTA), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), gentamicin sulphate, trypsin, insulin, streptomycin/penicillin and heparin were purchased from Sigma Company (St. Louis, Missouri, USA). Fetal bovine serum (FBS) and cell culture plates were ordered from Gibco Company (USA). CCl₄ was a product of National Pharmaceutical Group Chemical Reagent Co., Ltd., China. WST-1 was purchased from Beyotime Institute of Biotechnology, Haimen, China. *S. chinensis* extract (SCE) containing schizandrin A, schizandrin B and schizandrol A, was a commercial product obtained from Nantong Sihai Plant Extracts Co., Ltd., China.

Fish

Common carp (*Cyprinus carpio*) was obtained from the Freshwater Fisheries Research Centre of Chinese Academy of Fishery Sciences, Wuxi, China. Fish were reared at 26°C in a recirculation system and fed *ad libitum* twice a day with commercial diets containing approximately 40% crude protein, 10% crude lipid, 10% ash and an energy content of 21 kJ g⁻¹ DM. They were about 6 months old at the start of the experiment, with an average weight of 150 g.

Isolation and culture of hepatocytes

Fish hepatocytes were prepared according to the methods of Smeets et al. (1999) and Wan et al. (2004), with several modifications. Fish were anaesthetized in 0.05% tricaine methane sulphonate and sanitized with 70% alcohol, and blood was cleared from the caudal vein. An incision was made along the ventral midline from the vent to the gill isthmus. Two lateral incisions were made in the right ventral quadrant of the peritoneal cavity just anterior to the pelvic girdle and along the posterior margin of the gill operculum. The muscle and skin flap were removed, exposing the internal organs. Liver was then taken into a petri dish and washed with sterilized water and a Ca²⁺- and Mg²⁺-free buffer solution (pH 7.5) containing 0.145 M NaCl, 5.4 mM KCl, 5 mM EDTA, 1.1 mM KH₂PO₄, 12 mM NaHCO₃, 3 mM NaH₂PO₄, 100 mM HEPES. The liver tissue was then minced into pieces and digested in a solution of 0.25% trypsin (1:20 w/v) for 30 min at room temperature. The mixture was further trypsinized on a shaker at 200 rpm for 5 min to obtain the cell suspension which was then filtered through a 70-mesh sieve. The cell suspension was then centrifuged at 100 g for 2 min and the cell pellet was washed 3 times with L-15 culture medium (pH 7.4) containing 14.3 mM NaHCO₃, 20 mM HEPES, 50 µg ml⁻¹ gentamicin sulphate, 1 mM insulin, 10 mM hydrocortisone, and 2% (v/v) FBS. The cell suspensions were pooled and centrifuged at 1000 rpm for 2 min, and the pellet was washed and resuspended in L-15 culture medium and counted. When viability was > 90% as assessed with Trypan blue exclusion, the cells were

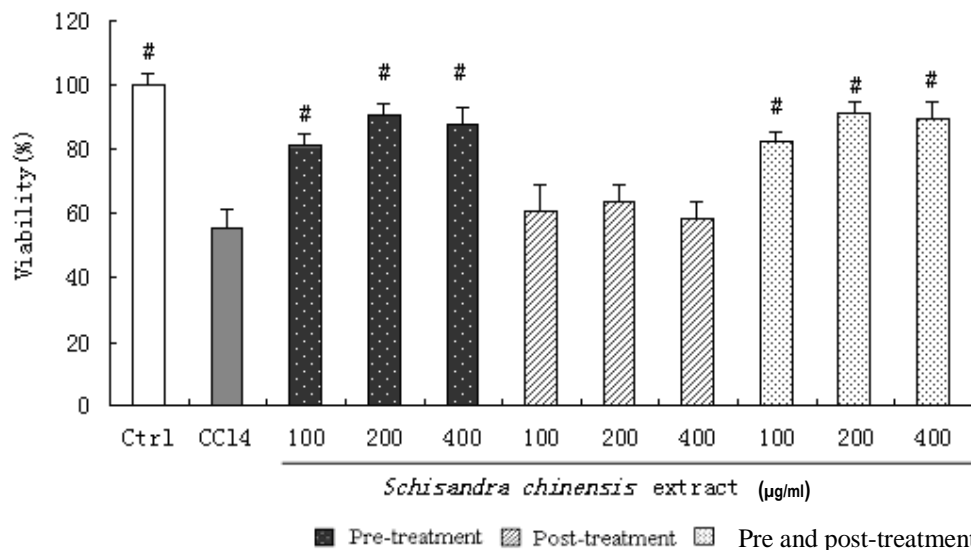


Figure 1. Effects of SCE on cell viability (percentage of control value) in CCl₄-treated primary hepatocytes. Values are expressed as mean \pm SD (n = 4). #P < 0.01, compared with cells treated with CCl₄ only.

used for experiments. Hepatocytes were adjusted to a density of 2.5×10^4 ml⁻¹ viable cells and plated in 96-well microplates (200 μ l well⁻¹) for viability assay or 2×10^5 ml⁻¹ viable cells and plated in 24-well microplates (600 μ l well⁻¹) for biochemical assays. The cells were cultured in L-15 culture medium supplemented with 1% streptomycin/penicillin and 10% FBS and kept for 24 h at 27°C under 5% CO₂ before the following experiments were conducted.

Treatments of hepatocytes with SCE

The hepatoprotective effect of SCE was investigated using an *in vitro* model of CCl₄-induced hepatocellular injury. After 24 h incubation, the cells were treated under the following three separate conditions:

1. Pre-treatment: The cells were pre-incubated with 0, 100, 200 and 400 μ g ml⁻¹ of SCE for 4 h before CCl₄ was added at a final concentration of 8 mM, the cells were then incubated with CCl₄ for 4 h.
2. Post-treatment: The cells were first incubated with CCl₄ at a concentration of 8 mM for 4 h and then SCE was added at concentrations of 0, 100, 200 and 400 μ g ml⁻¹. The cells were then incubated with SCE for 4 h.
3. Pre and post-treatment: The cells were first pre-incubated with 0, 100, 200 and 400 μ g ml⁻¹ of SCE for 4 h, then CCl₄ was added at a final concentration of 8 mM, after 4 h incubation with CCl₄, the cells were further treated with SCE at concentrations of 0, 100, 200 and 400 μ g ml⁻¹ for another 4 h.

For each set of conditions, four experiments were performed. Control (without adding CCl₄ and SCE), CCl₄ treatment and 3 concentrations of SCE treatment were set, each treatment was performed in quadruplicate. Before SCE or CCl₄ were added, the old medium should be completely removed and replaced with fresh medium containing SCE or CCl₄. At the end of each set of experiment, a 0.5 ml aliquot of supernatants from each individual well was collected in a 1.5 ml tube, centrifuged and stored at -20°C for various assays mentioned.

Parameter analysis

Viability of hepatocytes treated with SCE was measured using the WST-1 cell proliferation and cytotoxicity assay kit in accordance with the manufacturer's instructions. Briefly, 5×10^3 cells were cultured in 96-well plate, after pre-treatment, post-treatment or pre- and post-treatment of the cells with SCE, 10 μ l WST-1 was added to each well and the cells were incubated for an additional 2 h. The plate was shaken gently for 1 min before the absorbance of samples was measured under a wavelength of 450 nm using a microplate reader and the results were compared as percentages of control group. Lactate dehydrogenase (LDH), GPT, GOT, glutathione peroxidase (GSH-Px), superoxide dismutase (Donato et al., 2001) and malondialdehyde (MDA) in the supernatants were measured in a spectrophotometer (723C, Shanghai) using spectrophotometric diagnostic kits obtained from Nanjing Jiancheng Bioengineering Research Institute (Shen et al., 2009).

Statistics

The statistical analysis were performed with statistical package for social sciences (SPSS) software by one-way analysis of variance (ANOVA), followed by Tukey multiple comparison. *P < 0.05; #P < 0.01 were used as the criterion for significance.

RESULTS

Effects of SCE on cell viability in hepatocytes exposed to CCl₄

Cultured hepatocytes treated with CCl₄ showed a significant reduction of cell viability compared to the control (Figure 1). Pre-treatment and pre and post- treatment of the hepatocytes with SCE at all the three concentrations

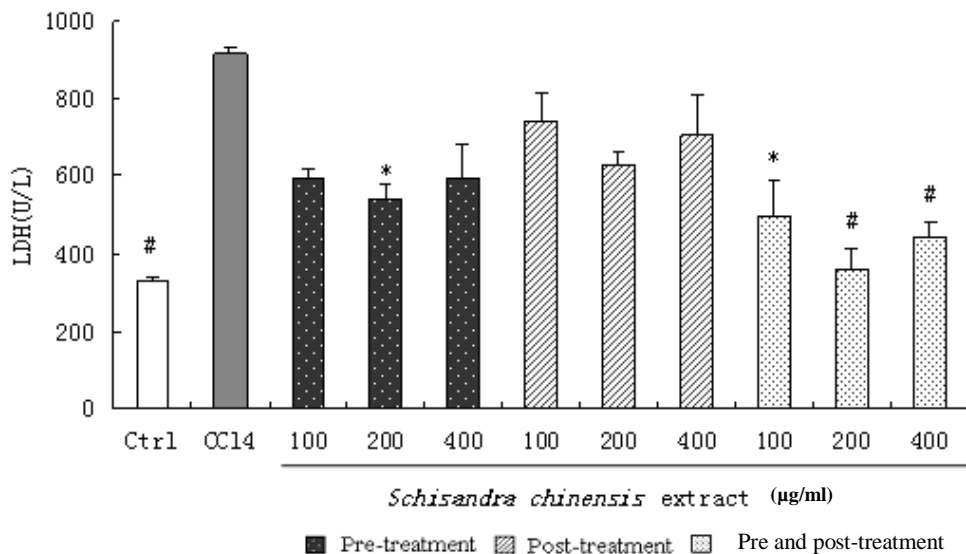


Figure 2. Effect of SCE on lactate dehydrogenase (LDH) in CCl_4 -treated primary hepatocytes. Values are expressed as mean \pm SD ($n = 4$). * $P < 0.05$; # $P < 0.01$, compared with cells treated with CCl_4 only.

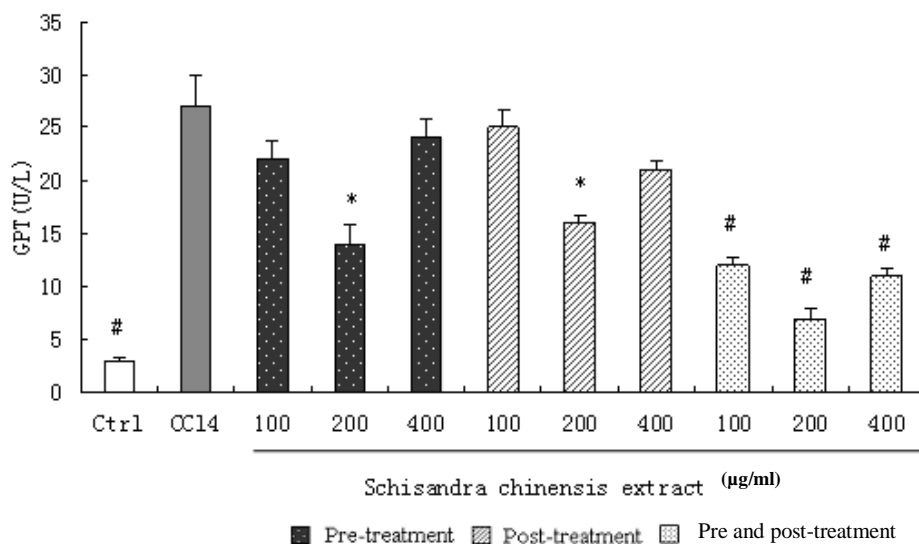


Figure 3. Effect of SCE on glutamate pyruvate transaminase (GPT) in CCl_4 -treated primary hepatocytes. Values are expressed as mean \pm SD ($n = 4$). * $P < 0.05$; # $P < 0.01$, compared with cells treated with CCl_4 only.

(100, 200 and 400 $\mu\text{g ml}^{-1}$) significantly enhanced the cell viability ($P < 0.01$). No significant difference was observed when the cells were post-treated with SCE.

Effects of SCE on LDH, GPT and GOT activities in hepatocytes exposed to CCl_4

Cultured hepatocytes exposed to CCl_4 showed a 3-fold increase of LDH (Figure 2), a 9-fold increase of GPT

(Figure 3) and a 6-fold increase of GOT (Figure 4) in the culture medium. Levels of all marker enzymes (LDH, GPT and GOT) increased significantly after the exposure of the hepatocytes to CCl_4 , as compared to the control. SCE pre-treatment (200 $\mu\text{g ml}^{-1}$) and pre and post-treatment (100, 200 and 400 $\mu\text{g ml}^{-1}$) of the hepatocytes caused significant decreases in the activities of LDH, GPT and GOT (Figures 2 to 4). Dose-dependent effects were observed, pre and post-treatment with 200 $\mu\text{g ml}^{-1}$ of SCE caused the most significant effects ($P < 0.01$) to reduce

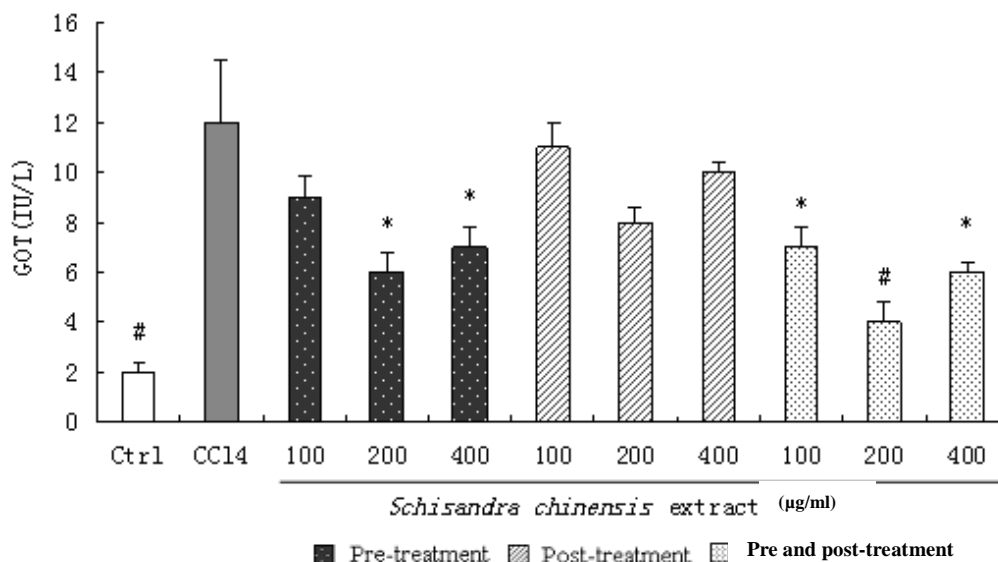


Figure 4. Effect of SCE on glutamate oxalate transaminase (GOT) in CCl₄-treated primary hepatocytes. Values are expressed as mean \pm SD (n = 4). **P* < 0.05; #*P* < 0.01, compared with cells treated with CCl₄ only.

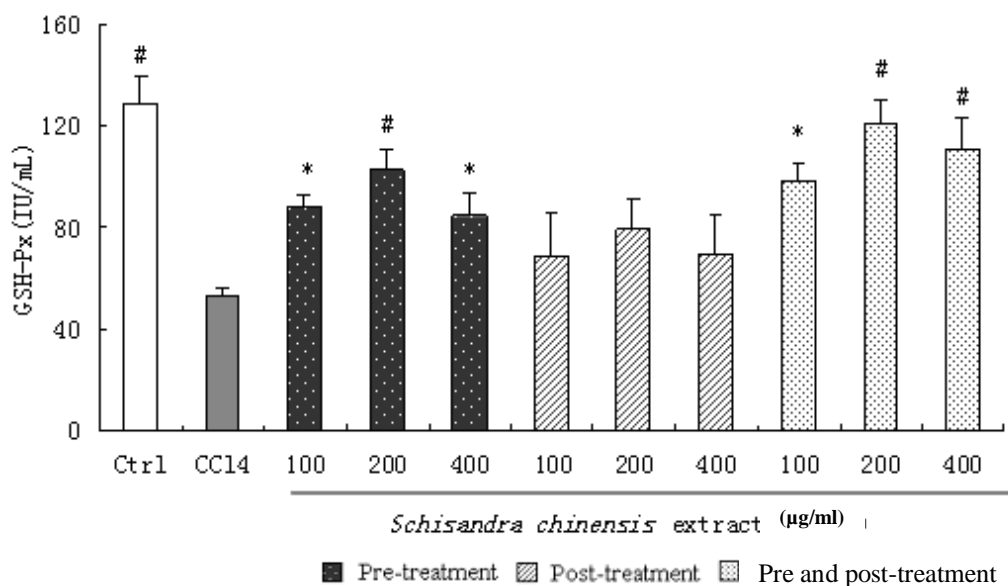


Figure 5. Effects of SCE on glutathione peroxidase (GSH-Px) in CCl₄-treated primary hepatocytes. Values are expressed as mean \pm SD (n = 4). **P* < 0.05; #*P* < 0.01, compared with cells treated with CCl₄ only.

the levels of LDH, GPT and GOT. However, post-treatments with SCE did not show any effects on the LDH and GOT activities (Figures 2 and 4). In the case of GPT activity, post-treatment of the cells with 200 $\mu\text{g ml}^{-1}$ of SCE still gave a significant effect, while no effects were observed when the cells were post-treated with 100 and 400 $\mu\text{g ml}^{-1}$ of SCE (Figure 3).

Effects of SCE on GSH-PX and SOD activities in hepatocytes exposed to CCl₄

The activities of GSH-Px (Figure 5) and SOD (Figure 6) were significantly decreased when hepatocytes were treated with CCl₄, as compared to the control. Pre-treatment and pre and post-treatment of the hepatocytes

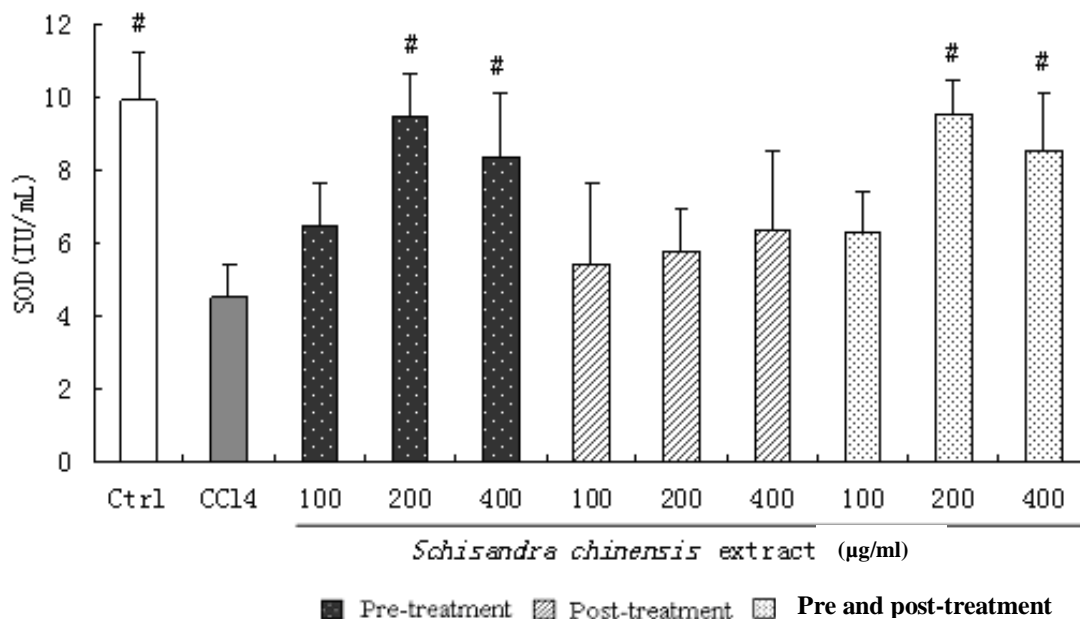


Figure 6. Effects of SCE on superoxide dismutase (SOD) in CCl₄-treated primary hepatocytes. Values are expressed as mean \pm SD (n = 4). #P < 0.01, compared with cells treated with CCl₄ only.

with SCE restored the activity of GSH-Px in all the tested three concentrations (Figure 5), while restoration of SOD activity was observed only when the hepatocytes were pre-treated or pre and post-treated with SCE at 200 and 400 $\mu\text{g ml}^{-1}$ (Figure 6). Post-treatment of the hepatocytes with SCE did not show any effects on the GSH-Px and SOD activities (Figures 5 and 6).

Effects of SCE on CCl₄-induced lipid peroxidation

Cultured hepatocytes treated with CCl₄ showed a 2.5-fold increase in the amount of MDA released into the medium (Figure 7). Pre-treating and pre and post-treating the cells with SCE at 200 and 400 $\mu\text{g ml}^{-1}$ significantly inhibited MDA formation, post-treating the cells with SCE did not show any effects on the MDA content (Figure 7).

DISCUSSION

Hepatotoxicity induced by CCl₄ is a commonly used model for the screening of hepatoprotective drugs (Gilani and Janbaz, 1995). The biochemical mechanism involved in the development of CCl₄ hepatotoxicity has long been investigated: it is now generally believed that the formation of reactive trichloromethyl radicals ($\cdot\text{CCl}_3$) from CCl₄ by CYP 450 is a crucial factor in the pathogenesis of CCl₄ hepatotoxicity (Ip and Ko, 1996). In the presence of oxygen, $\cdot\text{CCl}_3$ is quickly transformed into trichloromethyl peroxy radical ($\text{CCl}_3\text{O}_2\cdot$), $\text{CCl}_3\text{O}_2\cdot$ binds covalently to cellular proteins or lipids, and initiates the lipid perox-

idation in the cellular membrane (Levine and Reinhardt, 1983) resulting in the leakage of cellular enzymes (LDH, GPT and GOT) and finally cell apoptosis and necrosis (Manibusan et al., 2007). Therefore, cell viability and leakage of cytosolic enzymes (LDH, GPT and GOT) have been frequently used to assess the CCl₄ hepatotoxicity (Visen et al., 1998).

In the present study, the loss of cell viability and the significant elevated activities of LDH, GPT and GOT in the supernatants of the CCl₄-treated hepatocytes indicated the cellular leakage or hepatocyte damage. Pre-treatment or pre and post-treatment of the hepatocytes with SCE significantly increased the cell viability and decreased the activities of LDH, GPT and GOT, indicating that SCE could maintain the functional integrity of the hepatocyte membrane, protect the hepatocytes against CCl₄-mediated toxicity. Our results are consistent with studies on mammals both *in vitro* and *in vivo* that SCE was effective in reducing the increased activities of GPT, GOT and LDH induced by CCl₄ (Hancke et al., 1999; Qi et al., 2009).

MDA, a decomposition product of lipid hydroperoxides (Gutteridge, 1995) is widely used as marker of lipid peroxidation (Mansour, 2000), its elevated level could reflect the degree of lipid peroxidation injury in hepatocytes (Hu et al., 2001). In this study, MDA levels in the supernatant of CCl₄ treated hepatocytes were significantly elevated, pre-treatment or pre and post-treatment of the hepatocytes with SCE (200 and 400 $\mu\text{g ml}^{-1}$) significantly suppressed the elevation of MDA caused by CCl₄, indicating the anti-lipid peroxidation effect of SCE.

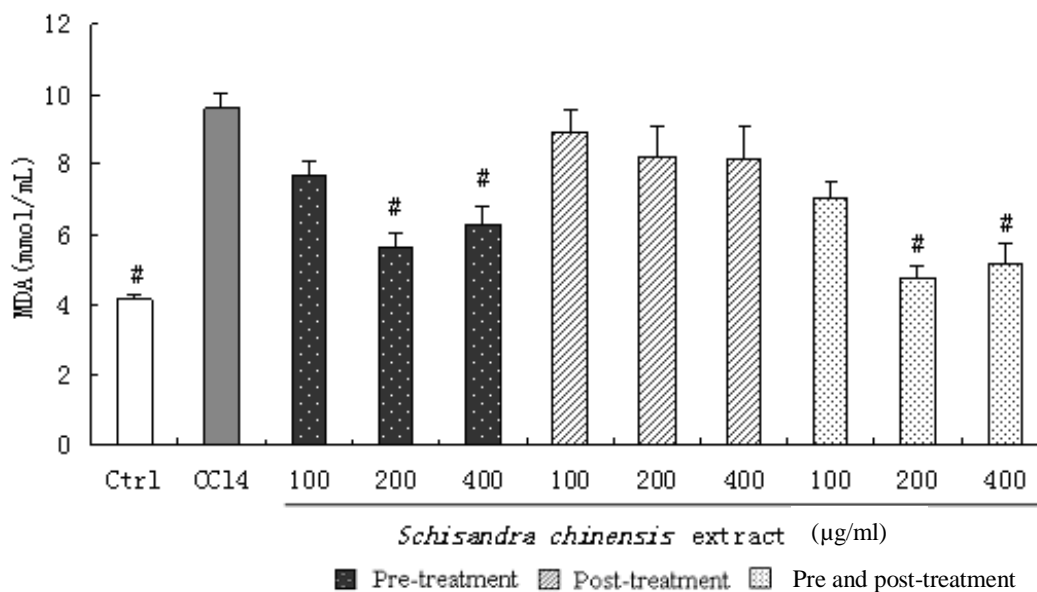


Figure 7. Effects of SCE on malondialdehyde (MDA) in CCl₄-treated primary hepatocytes. Values are expressed as mean \pm SD (n = 4). #P < 0.01, compared with cells treated with CCl₄ only.

Antioxidant property is claimed to be one of the mechanisms of hepatoprotective drugs (Recknagel, 1967). GSH-Px and SOD are two important antioxidant enzymes involved in enzymatic antioxidant defence mechanisms (Kalayci et al., 2005). It has been suggested that the lipid peroxidases generated after CCl₄ treatment is eliminated by GSH-Px in the presence of glutathione, thus curbing the propagation of lipid peroxidation (Koneri et al., 2008). The significant decreases of GSH-Px and SOD activities in CCl₄-treated hepatocytes in this study may partly explain the 2.5-fold elevation of MDA, while pre-treatment or pre- and post-treatment with SCE restored the GSH-Px and SOD activities (Figures 5 and 6), which may contribute to the suppressed lipid peroxidation as evidenced by the suppressed formation of MDA. The antioxidant effect of *S. chinensis* is attributed to its lignan constituents such as schisandrin B and schisanhenol (Hancke et al., 1999), which increased superoxide dismutase and catalase activities in rat liver cytosol (Johnston and Santillo, 2002), inhibited the lipid peroxidation measured by means of MDA formation induced by iron/cysteine in rat liver microsomes (Hahn, 2002).

Comparing the pre-treatment with the post-treatment regimen, we found that pre-treatment with SCE showed the protective effect against CCl₄-mediated toxicity, while post-treatment did not show significant effects on all the tested parameters except GPT; this may suggest that SCE can be potentially used for preventing rather than curing liver diseases in fish. The present findings demonstrated the hepatoprotective effect of SCE against hepatocyte damage induced by CCl₄ in fish. The hepatoprotective activity of SCE may be attributed to the

enhancement of the hepatic antioxidant system. Further *in vivo* studies may provide better understanding of SCE as a hepatoprotective agent potential for the prevention of hepatobiliary syndrome in fish.

ACKNOWLEDGMENTS

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Full Length Research Paper

Irritant effects of *Euphorbia prostrata*

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Irritant dermatological investigations of locally occurring *Euphorbia prostrata* were carried out to discover the irritant principle present in it. For this purpose, successive solvent extraction was carried out by using polar, non polar as well as intermediate polarity solvents for the extraction of active irritant compounds from the whole herb. Irritant potentials of these extracts were evaluated on rabbit's skin. Biological assay showed that only the chloroform extract was active because it produced irritancy at a certain dose level. Five fractions Ep-1 to Ep-5 were isolated from the active chloroform extract of the plant by column and thin layer chromatography. Irritant dermatological prospective of these fractions were also evaluated on rabbit's ear skin. Out of the five isolated fractions, Ep-2 and Ep-4 appeared to be the strong irritant fractions, while Ep-1, Ep-3 and Ep-5 proved to be moderately irritant fractions at the same dose level. A possible structure-activity relationship of these active fractions was also discussed based on the ultraviolet (UV) and infrared (IR) spectrum.

Key words: Irritancy, Euphorbiaceae, chromatography.

INTRODUCTION

Euphorbia prostrata is a weed; it grows in the cultivated fields of wheat, rice or other crops and also in the gardens along with grass. It is commonly observed that during the removal of this weed, irritation on the hands of workers often occur. The character and uniqueness of such irritant compounds present in this *Euphorbia* species and its mechanism of action has not been previously investigated. Taking into account all these observations, the current study of irritant contact dermatological studies of *E. prostrata* was conducted. *E. prostrata* belongs to the family Euphorbiaceae; a large family of Dicotyledonous Angiosperms (Charles et al., 2007). Family Euphorbiaceae is widely distributed throughout both hemispheres and ranges in morphological form from largest dessert succulents to trees and even small herbaceous types (Cutler et al., 1987).

E. prostrata is native to West Indies, but is now widely distributed throughout the tropics and subtropics (Turner,

1995). It occurs throughout tropical Africa and the Indian Ocean Islands (Singh, 1994). *E. prostrata* grows in gardens, on distributed grounds, in cultivated land and roadside, especially in sandy soils, from sea-level to high altitude (Carter and Leach, 2001). It is also found throughout India as a weed in the plains and at lower elevations (Nguyen and Sosef, 1999). The major constituents include anthraquinone glycosides, flavanoids, phenols, phlobotannins, polysaccharides, saponins, tannins and terpenoids. Alkaloids were not present in very high amounts (Singla and Pathak, 1991). A range of hydrolysable ellagitannins were isolated, including prostratin A, B, and C, euphorbins G and H, tellimagradin I and II, rugosin A, D, E and G from different fractions of extracts of the dried leaves. Flavanoids isolated from the aerial parts include: kaempferol, cosmosin (apigenin-7-glucoside), rhamnatin-3-galactoside, quercitin and quercitin-3-rhamnoside. Other constituents of the aerial parts include the sterols β -amyryne acetate,

β -sitosterol, campesterol, stigmasterol and cholesterol. Aerial parts also contain the terpene alcohol β -terpenol, gallic acid, corilagin, 1, 2, 3-tri-O-galloyl-D-glucose, geraniin, and various amino acids, including n-valeramide and N, N-dimethyl-4-benzoxybutylamine. Roots contain a myricyclic alcohol and two triterpenes, taraxerol and tirucalol (El-Mahy, 2004; Yoshida et al., 1990).

All parts of *E. prostrata* are used as traditional medicine around the globe. Its leaves are used as antidote for stings especially of wasp and scorpion sting. This weed is also used as anti abortive agent and for painful menstruation. In Uganda, pregnant women eat the boiled shoots, mixed with sesame to reduce the risk of miscarriage (Neuwinger, 2000; Kamatenesi-Mugisha and Oryem-Origa, 2007; Kokwaro, 1993; Ogwal, 1996). Leaf powder mixed with palm oil is rubbed on the head to treat headache. Crushed whole plant was eaten with bread against kidney stones. Around the Indian Ocean Islands, an infusion of the leaves or aerial parts was taken either alone or combined with other plants to treat diarrhea, dysentery and stomachache. It showed activity against *Shigella dysenteriae* type I induced diarrhea in rats (Watt and Breyer-Brandwijk, 1962; Kamgang et al., 2007). *E. prostrata* showed antibacterial activities as well as inhibitory effects against HIV-1 protease and hepatitis C virus protease (Hussein et al., 1999, 2000). It possessed anti-fungal activity against certain dermatophytes in experimentally infected animals (Pal and Gupta, 1979). In India, the latex was used to treat diabetes, as it was considered to have hypoglycaemic activities (Akhtar et al., 1984). Many species of family Euphorbiaceae are commonly used as sources of medicines by various tribal and ethnic communities in Pakistan.

MATERIALS AND METHODS

Plants

E. prostrata plants were collected from the Botanical Garden, Government College University, Lahore and from different areas around and within Lahore. These were authenticated by Dr. Sultan, Herbarium, Department of Botany, Government College University, Lahore against specimen number G. C. Herb. Bot. 605. The herbaceous plants were dried under the shade at room temperature for about ten days. The dried plants were then pulverized to fine powder and stored in black polythene bags.

Instruments

Instrument used in this research were distillation apparatus (Quick fit, England), electric balance (Sartorius), oven (Mettler, W. Germany), water bath, Soxhlet apparatus.

Chemicals

All the chemicals used were of BDH analytical grade. The following chemicals were used, which were purchased from local market, H₂SO₄, acetic anhydride, aluminium chloride, anisaldehyde, vanillin, iodine, sodium sulphate (anhydrous).

Solvents

All the solvents used were of BDH analytical grade. The following solvents were commonly used, which are purchased from local market. All the solvents were re-distilled before use: petroleum ether (40 to 60°C), chloroform, ethanol, methanol, glacial acetic acid, acetone, dichloromethane, hydrochloric acid, acetic acid, distilled water.

Chromatographic material

Chromatographic materials used were, silica gel 60 (70-230 mesh ASTM) for column Chromatography – by E. Merck (Germany), Silica gel G₆₀ (HF 254) for thin layer chromatography by – E. Merck (Germany).

Spectral analysis

The ultra violet spectra were recorded on UV-2450 SHIMADZU spectrophotometer by using chloroform as solvent. The infrared spectra were measured on IRPRESTIGE-21 SHIMADZU spectrophotometer using thin film on NaCl disc by mull method.

Micro capillaries

10 μ l microcapillaries (Doumond Microcaps, U.S.A) were used for topical application of the materials on the skin of the animals.

Animals

The study was carried out as per approved protocol by the Animal Ethics Committee, University College of Pharmacy, University of the Punjab, Lahore, Pakistan. Healthy adult male/female rabbits of albino strain of species *Oryctolagus cuniculus* and subsp. 18 rabbits weighing 1.0 to 1.5 kg were purchased from local market. All the rabbits were acclimatized in the animal house of University College of Pharmacy, University of the Punjab, Lahore for a period of three days and were provided with carrots, fresh green fodder and tap water *ad libitum*.

Vehicle

Unless otherwise stated, redistilled acetone was used as a vehicle for bioassay.

Solvent extraction

The pulverized dried *E. prostrata* plants (600 g) were extracted successively in petroleum ether (40 to 60°C), chloroform and methanol by using 2.0 L of each solvent for soaking. Maceration was carried in each solvent for 4 days at room temperature (25 \pm 2.5°C). The solvent of each extracted material was removed under reduced pressure and the residues were weighed.

Thin layer chromatography (TLC)

20 \times 5 cm glass plates were used for this purpose. 30 g silica gel G₆₀ was made into slurry by mixing with 90 ml of distilled water and spread uniformly on 21 plates of 20 \times 5 cm size with the help of moving spreader Dosga applicator, which was already adjusted at 0.25 mm. The plates were dried at room temperature and activated

Table 1. Grading of irritant reactions. Adopted from Hacker (1971).

Reaction grades	Explanation
-	No reaction
±	Doubtful reaction, diffused inflammation with no clear visible symptoms
+	Slight reddening of the main vessels without reddening the area in between
++	Marked reddening of the main vessels with reddening of the areas in between
+++	Intense reddening of the entire ear often accompanied with macroscopic visible hyperplasia
++++	Visible exudative lesion with marked epidermal damage

in an oven at 100°C.

Spotting on plates

Small quantity of liquid extract was taken and spotted on layers at 1.5 cm above the baseline of the plate with the help of microcapillary tube. The distance between the two spots was kept at 1.5 cm.

Development of plates

The spots on thin layer plates were dried by air-dryer and developed in chromatographic jars. Inner side of the tank was made saturated with the solvent, after attaching the solvent soaked filter paper in it. 30 to 35 ml of solvent was poured into the tank, so that it rises 1 to 2 cm above from bottom of the tank. The plates were placed inside the tank and the lid was closed. The plates were allowed to develop. The solvent was run up to 1.5 cm from the upper edge of the plates. Solvent front was marked and after drying the plates with air-dryer, the spots were detected under UV light and with iodine. Different solvent systems were used for TLC of three solvent extracts that is, petroleum ether extract, chloroform extract and methanol extract.

Column chromatography

The active extract was further subjected to column chromatography. Different fractions were obtained from the column by monitoring with thin layer chromatographic procedure. Similar fractions were pooled and five isolated fractions were obtained at the end, which were subjected to main assay for irritancy.

Biological assay for irritancy

Dilution series

10 mg of the dried extract was accurately weighed by using electric balance and made up to 10 mg/10 ml (w/v) solution with acetone. The dilution series was prepared according to the equation, as was given by Evans and Schmidt (Schmidt and Moul, 1983).

$$C_m = C_o \times a^{-m}$$

Where: C_o = Initial concentration; C_m = concentration after m dilution; a = dilution factor. The dilution factor in all the cases was kept at 2, and six or seven dilutions were prepared.

Pilot assay

The biological assay for irritancy was adopted from Evans and Schmidt's method (Evans and Schmidt, 1979). 20, 40, 80 and 120

µl solutions from each dilution were applied to the inner surface of rabbit's ear and the untreated ear was used as control. The ears were examined for redness after 30 min of application and according to time mentioned in Table 2, until two examinations indicated that further redness would not occur. Time for maximum erythema was noted. Four dilutions were chosen for the main assay to include one dilution that will give maximum positive response. The animals were also examined after 24 and 48 h to ascertain the chronic inflammatory dose. The degree of redness corresponding to "++" was noted giving result corresponding to "IU" that is, irritant units of Hacker (1971) and cited by Evans and Schmidt (Evans and Schmidt, 1979). If no redness was observed, the assay was repeated by using more concentrated solution of the extract on new rabbit's ear.

Main assay

For the main assay, a group of 6 rabbits for each dilution was used. 20 µl of the most diluted solution of the chosen series was applied to one of the ears of rabbit in that group by using 10 µl microcapillaries. The animals of other dilution group were also treated similarly by increasing concentration of irritants. Rabbits were examined after 30 min of application and then after 30 min intervals. The number of ears showing marked inflammation of the major blood vessels was recorded. ID_{50} that is, irritant dose in 50% individuals was taken as a dose corresponding to the 50% cumulative frequency. The evaluation of irritant response has been described in Table 1. The dose causing an ear redness to the degree ++ is defined as IU (Hacker, 1971) and expressed in µg/ml per ear.

RESULTS AND DISCUSSION

Solvent extraction

Successive extraction with petroleum ether, chloroform and methanol showed that the percentage yields of extracts of petroleum ether, chloroform and methanol were 2.95, 4.07 and 13.38%, respectively.

Thin layer chromatography

TLC results of petroleum ether, chloroform and methanol extracts of whole herbs of *E. prostrata* showed that following were the solvent systems which resolved the extracts into maximum components. Petroleum ether extract was resolved into five components by solvent system; petroleum ether/chloroform with ratios 95:5, 95:7

Table 2. Irritant response of the crude solvent extracts of *Euphorbia prostrata* on rabbit's ear.

Dose ($\mu\text{g}/\mu\text{l}$)	Extracts	Response after – acute time (h)										Chronic time (h)		
		1	2	3	4	5	6	7	8	9	10	24	48	72
20/20	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
40/40	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
80/80	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	±	+	++	++	+	+	+	±	±	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
120/120	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	+	+	++	+++	+++	+++	+++	++	++	+	+
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-

and 95:10. Chloroform extract was resolved into six components by solvent systems; petroleum ether/chloroform/methanol with ratios 20:80:3 and 20:80:5. Methanol extract was best resolved into four components by solvent system; chloroform/methanol with a ratio of 90:10.

Isolated compounds

Chloroform extract of the plant was subjected to column chromatography, and five different compounds were isolated from the extract namely: Ep-1, Ep-2, Ep-3, Ep-4 and Ep-5.

Spectral analysis

Compounds Ep-1

The ultraviolet and infrared spectrums of the isolated compounds were obtained and are shown in Figures 1 to 10.

Irritancy assay

The results of preliminary irritant responses of crude extracts of *E. prostrata* on rabbit's ear have been outlined in Table 2. The results of irritant reactions of isolated compounds of chloroform extract of *E. prostrata* on rabbit's ear have been outlined in Table 3, while the irritant results of individual isolated compounds (Ep-1, Ep-2, Ep-3, Ep-4 and Ep-5) at four different doses have been outlined in Tables 4 to 8.

DISCUSSION

For isolation of phytochemical fractions from this species, successive solvent extraction was carried out. For this purpose, both non-polar and polar solvents that is, petroleum ether (40 to 60°C), chloroform and methanol were used (Brain and Turner, 1975). The solvent extraction procedure was based on the assumption that petroleum ether (40 to 60°C) was a non-polar solvent; it probably extracted the least polar compounds. Methanol on the other hand was a polar solvent, and extracted most of the polar components from the crude powder. Chloroform possessed an intermediate polarity and probably extracted the compounds with intermediate polarities (Singla and Pathak, 1989). The dried powder of whole herb (600 g) of *E. prostrata* was thus subjected to successive extraction in these three common solvents under the laboratory conditions. Results of the broad solvent extraction in the form of percentage yield indicated that the production of both the polar and non-polar components were not equal. Out of the three types of extracted materials, the polar components (13.37%) were extracted in methanol and were in higher yield than others. The components with intermediate polarity (4.07%), which were extracted with chloroform, were next in the yield. On the other hand, the non-polar components (2.95%) which were extracted in petroleum ether were in the lowest yield. It could thus be concluded that the powdered *E. prostrata* contained high proportion of polar compounds than others.

All of the three extracts were subjected to a comparative TLC analysis using different solvent systems in each case. The main purpose of this analysis was to have an idea of the total number and chromatographic behavior of the compounds present in each extract. The

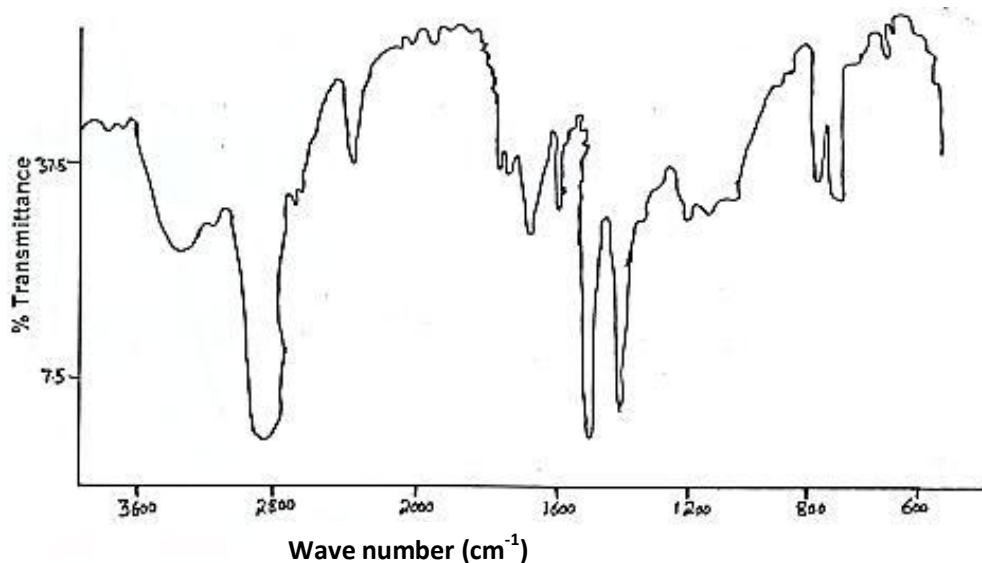


Figure 6. Infrared spectrum of Ep 3.

Table 7. Irritant response of the isolated compound Ep-4 from chloroform extract of *Euphorbia prostrata* on rabbit's ear.

Dose levels ($\mu\text{g}/\mu\text{l}$)	Response after – Acute time (h)												Chronic time (h)		
	1	2	3	4	5	6	7	8	9	10	11	12	24	48	72
120/120	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++
80/80	+	++	++	++	++	+++	+++	+++	+++	++	++	++	++	++	++
40/40	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-
20/20	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-

Table 8. Irritant response of the isolated compound Ep-5 from chloroform extract of *Euphorbia prostrata* on rabbit's ear.

Dose levels ($\mu\text{g}/\mu\text{l}$)	Response after – Acute time (h)												Chronic time (h)		
	1	2	3	4	5	6	7	8	9	10	11	12	24	48	72
120/120	++	++	++	++	++	+++	+++	+++	+++	++	++	++	++	++	+
80/80	+	+	+	+	++	++	++	++	++	++	++	++	++	++	++
40/40	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
20/20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

results of this analysis showed that the best solvent system which resolved the mixture of petroleum ether extract into five components seemed to be petroleum ether/chloroform (with ratios 95:5, 95:7 and 95:10). Chloroform extract was segregated maximum into six components by the petroleum ether/chloroform/methanol (20:80:3 and 20:80:5). On the other hand, the mixture of polar components present in the methanol extract was best resolved into four components by chloroform/methanol (90:10).

Preliminary irritancy assay was performed with all three types of solvent extracts and the isolated compounds of *E. prostrata* on rabbit's ear. This method was originally

innovated by Hecker (1971) for evaluating the irritant principles from croton oil on mice's ears, which was later on followed with minor modifications by Evans and Schmidt (1980) and Evans and Soper (1978), for evaluating the irritancy of tigliane, daphnane and ingenane series of diterpene esters from various *Euphorbia* species. These authors used albino mice as an animal model for their investigations. In the present work, the same method was used, but instead of mice, rabbits were used as animal model to assess the irritancy activities. Although the erythema produced by these extracts/isolated compounds was of weak and diffused type, but could easily be evaluated. Many other authors

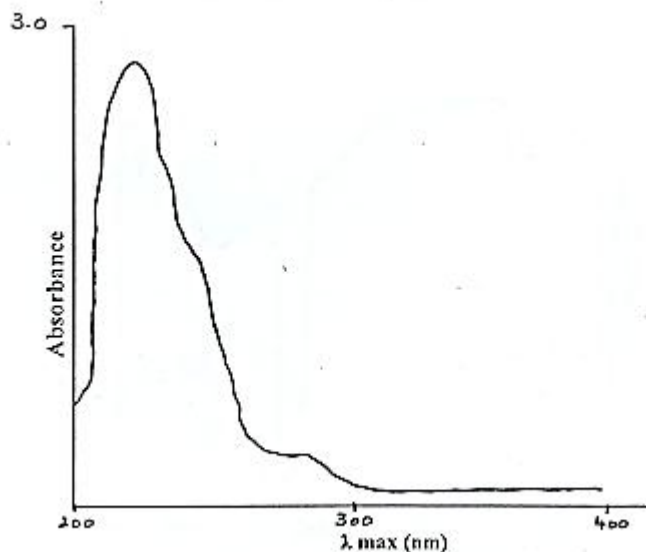


Figure 7. Ultra violet spectrum of Ep 4.

used albino rabbits instead of mice for similar purpose (Anderson et al., 1987; Benazra et al., 1985).

Results indicated that all three solvent extracts exhibited no irritant responses when the low doses of 20 and 40 μg were used but at higher doses of 80 and 120 μg , only chloroform extract produces redness of +, ++ and +++ intensity on rabbit's ears. Hence from all the three extracts, chloroform extract seemed to be irritant than other two extracts at this dose level. Methanol and petroleum ether extract seemed to be inert in its irritant reaction with all the four doses used. It could thus be concluded that intermediate polarity constituents of *E. prostrata* were responsible for such adverse reaction on the animal's skin (Table 2).

The chloroform extract of *E. prostrata* was further subjected to column chromatographic analysis to isolate the active irritant compounds, using an increasing quantity of methanol in chloroform. The elution process was monitored by silica gel thin layer chromatography. Thirteen pooled fractions were obtained. All the pooled column fractions gave irritant reactions, but level of irritation intensity is variable with reference to time.

Further, five fractions were isolated from the first, third, sixth, eleventh and thirteenth pooled column fractions of the chloroform extract of *E. prostrata* on the basis of their superior irritation activity of +++ (intense reddening of the entire area). They were named as Ep-1 to Ep-5.

Isolated fraction Ep-1 was isolated from the first column fraction and was brown in color. The thin layer chromatography of this fraction indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 40:60) was used. It gave light yellow colour with iodine. Under UV, the spot appeared light pink fluorescence. The compound, Ep-1, had strong absorption at $\lambda_{\text{max}} = 252 \text{ nm}$ (Figure 1) which was probably due

to $n \rightarrow \pi^*$ transition. This value is assigned to aldehydes. It also indicated the presence of conjugated polyenes Me (C \equiv C)Me.

The infrared spectrum of Ep-1 (Figure 2) showed strong and broad absorption band between 3600 to 3000 cm^{-1} due to -OH absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. The stronger band at 2900 cm^{-1} showed the C-H stretching present in alkane/alkene and alkynes. Often, such bands are shown by the methyl, methylene or aryl groups resulted from symmetrical or asymmetrical stretching C-H modes. The weak absorption at 2700 cm^{-1} was possibly due to presence of some bands -NH₂, =NH or $\equiv\text{N}$. This indicated the presence of amyl/amines or ammonium. Bands in this region were due to N-H stretching. The band at 2350 cm^{-1} was a weak absorption and was possibly due to the presence of carboxylic group present in compound. The weak absorption at 1950 cm^{-1} possibly indicated the presence of some cyanates. The medium band at 1740 cm^{-1} indicated carboxyl absorption possibly due to C=O stretching vibration. Such vibration was present in keto esters, six ring or lactones. Absorption at 1660 cm^{-1} was probably due to β -keto esters in H-bonding in enol form. Strong peak at 1460 cm^{-1} also indicated the -C-N= stretching vibrations possibly due to -NH₂, =NH or $\equiv\text{N}$ absorption. The absorption at 1390 cm^{-1} was probably due to the presence of phosphates in the compound. The medium absorption at 720 cm^{-1} indicated the presence of 5 adjacent H and monosubstituted benzene ring (Williams and Fleming, 1980). The available spectral evidence showed that Ep-1 was probably a compound containing aldehyde, amide, alcoholic, phenolic, cyanate and lactone ring. It also indicated the presence of phosphates in the compound.

Isolated fraction Ep-2 was isolated from the third column fraction. It was a brown fraction. Thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 40:60) was used. It gave yellow colour with iodine. Under UV, the spot appeared light pink fluorescence.

The fraction, Ep-2 had strong absorption at $\lambda_{\text{max}} = 250 \text{ nm}$ (Figure 3) which was probably due to $n \rightarrow \pi^*$ transition. This value is assigned to unsaturated acids/esters, nitriles or amides. The infrared spectrum of Ep-2 (Figure 4) showed broad and medium absorption band between 3600 to 2930 cm^{-1} due to -OH absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. Stronger band at 2900 cm^{-1} showed the C-H stretching vibration present in alkane/alkene or alkynes. Often, such bands are shown by the methyl/methylene or aryl groups resulted from symmetrical or asymmetrical stretching C-H modes. The

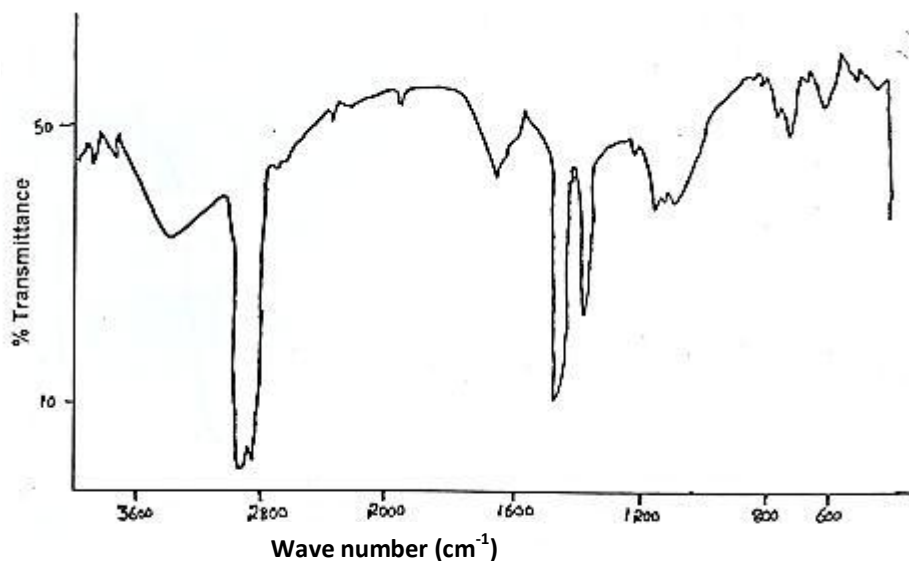


Figure 8. Infrared spectrum of Ep 4.

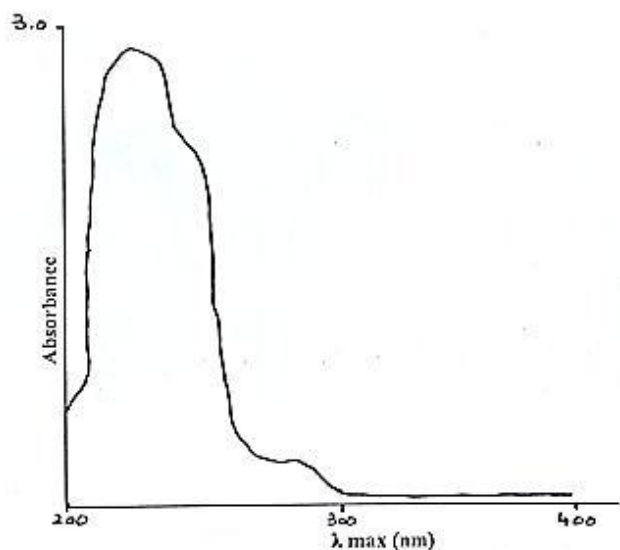


Figure 9. Ultra violet spectrum of Ep 5.

strong absorption at 2840 cm^{-1} was possibly due to the C-H stretching present in aryl esters ($\text{C}-\text{O}-\text{CH}_3$). Weak absorption at 1950 cm^{-1} possibly indicated the presence of some cyanates. Strong absorption at 1680 cm^{-1} was probably due to presence of $> \text{C}=\text{N}$ - present in imines. Absorption at 1470 cm^{-1} was a medium band and possibly indicated the presence $\text{CH}_2=\text{CH}_3$ - groups which was present in the alkanes or alkenes. Medium absorption at 1350 cm^{-1} was probably due to presence of nitrates in the compound. Absorption at 1080 cm^{-1} was a weak band and indicated presence of $\equiv\text{C}-\text{O}-\text{C}\equiv$ present in ether (Williams and Fleming, 1980). The available spectral

evidence showed that Ep-2 was probably a compound containing alcoholic/phenolic/carbonyl/alkane/alkene/alkyne/cyanate/s/nitrites or ether.

Isolated fraction Ep-3 was isolated from the sixth column fraction. It was light yellow fraction. The thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 40:60) was used. It indicated light yellow colour with iodine. Under UV, the spot appeared light pink fluorescence. The fraction, Ep-3 had strong absorption at $\lambda_{\text{max}} = 242\text{ nm}$ (Figure 5) which was probably due to $n \rightarrow \pi$ and $\pi \rightarrow \pi$ transition. This absorption perhaps indicated some double bonds in the molecule. The infrared spectrum of Ep-3 (Figure 6) showed broad and medium absorption band between 3300 to 3050 cm^{-1} due to $-\text{OH}$ absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water molecule incorporated during the recording of spectrum. Broad and strong band at 2920 to 2820 cm^{-1} indicated stretching vibration in plane possibly due to $\equiv\text{CH}=\text{CH}_2$ or $-\text{CH}_3$ groups present in the molecule. Weak absorption at 2710 cm^{-1} was possibly due to presence of overtone bands $-\text{NH}_2$, $=\text{NH}$ or $\equiv\text{N}$ stretching vibrations possibly due to presence of some amyl/amines, or ammonium. Medium absorption at 1730 cm^{-1} possibly indicated the presence of keto esters/six ring or larger lactones. Medium absorption at 1450 cm^{-1} possibly indicated presence of $\text{CH}_2=$, CH_3- groups which was present in the alkanes or alkenes. The strong absorption at 1380 cm^{-1} was probably due to presence of nitro ($\text{C}-\text{NO}_2$) and methyl ($-\text{CH}_3$) in the compound. Medium absorption at 1140 cm^{-1} indicated presence of sulphur group ($=\text{SO}_2$). Medium absorption at 690 cm^{-1}

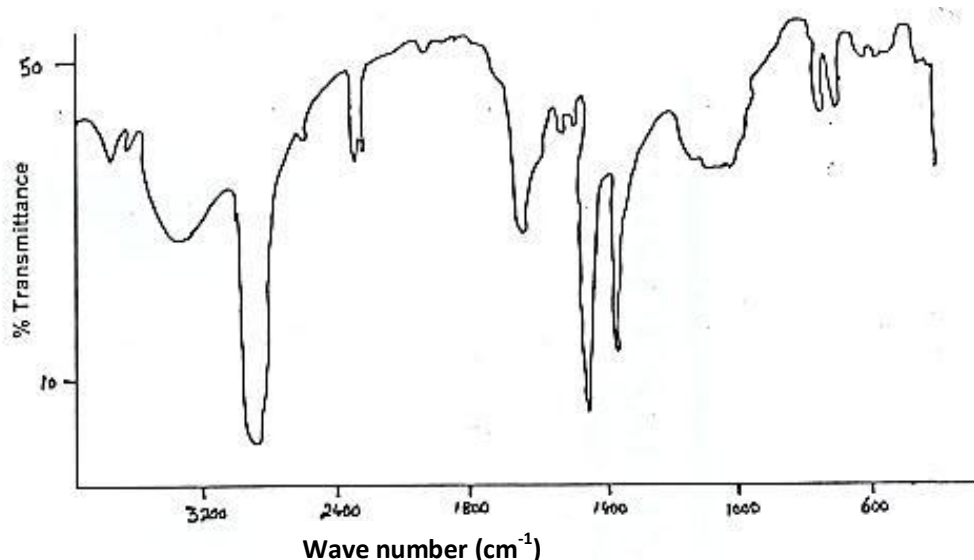


Figure 10. Infrared spectrum of Ep 5.

possibly indicated the presence of halogen groups (Williams and Fleming, 1980). The available spectral evidence showed that Ep-3 was probably a compound containing alcoholic/phenolic/carbonyl/methyl/alkane/alkene/aryl/amines/nitro/ammonium/keto esters/halogen groups and 6 ring lactone. It also indicated the presence of sulphur in the compound.

Isolated fraction Ep-4 was isolated from the eleventh column fraction. It was a dark brown compound. The thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 95:3) was used. It gave light yellow colour with Iodine. Under UV, the spot appeared as a light pink fluorescence. The fraction, Ep-4, had strong absorption at $\lambda_{\max} = 230$ nm (Figure 7) which was probably due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transition. This absorption perhaps indicated some double bonds in the molecule. The infrared spectrum of Ep-4 (Figure 8) showed strong and broad absorption band at 3500 to 3060 cm^{-1} due to $-\text{OH}$ absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. The strong and broad band at 2970 to 2840 cm^{-1} indicated alkane/alkene/alkynes or oximes. Weak absorption at 1670 cm^{-1} was probably due to stretching vibration of carboxylic acid/ketones or aldehyde. The strong absorption at 1450 cm^{-1} possibly indicated the stretching vibrations of $-\text{NH}_2/\text{=NH}/\equiv\text{N}$ or $-\text{NH}_2\text{CO}/\text{=NHCO}/-\text{CN}$. Medium absorption at 1360 cm^{-1} was probably due to presence of nitrates in the compound. Weak absorption at 1080 cm^{-1} probably indicated the deformation of $\equiv\text{CH}$, $=\text{CH}_2$ or CH_3 (Williams and Fleming, 1980). The available spectral evidence showed that Ep-4 was probably a

compound containing alcoholic, phenolic, carbonyl, alkane, alkyne, oximes, esters, amide or some nitrates.

Isolated fraction Ep-5 was isolated from the thirteenth column fraction. It was a dark brown compound. The thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 95:3) was used. It gave yellow colour with Iodine. Under UV, the spot appeared yellow. The fraction, Ep-5 had strong absorption at $\lambda_{\max} = 240$ nm and a weak absorption at 284 nm (Figure 9). Both the strong and weak absorptions are probably due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transition. The infrared spectrum of Ep-5 (Figure 10) showed strong and broad absorption band between 3500 to 3200 cm^{-1} due to $-\text{O}-\text{H}$ stretching vibration possibly due to some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. The strong and broad band at 2980 to 2820 cm^{-1} showed the C—H stretching present in alkane/alkene or alkynes. Often, such bands are shown by the methyl/methylene or aryl groups resulted from symmetrical or asymmetrical stretching C-H modes. Weak absorption at 2700 cm^{-1} was possibly due to presence of overtone bands $-\text{NH}_2/\text{=NH}/\equiv\text{N}$. This indicated the presence of aryl/amines or ammonium. Bands in this region were due to N—H stretching. The weak band at 2320 cm^{-1} was possibly due to the presence of carboxylic group present in compound. Weak absorption at 1670 cm^{-1} was probably due to H-bonding in enol form. The strong absorption at 1450 cm^{-1} possibly indicated presence of alkane/alkene or alkyne ($-\text{CH}_3$, $=\text{CH}_2$, $\equiv\text{CH}$). A medium absorption at 1375 cm^{-1} was probably due to the presence of sulphur in the compound. The absorption at 700 cm^{-1} was a weak band and indicated the presence of some halogen groups (Williams and Fleming, 1980).

The available spectral evidence showed that Ep-5 was probably a compound containing amide/alcoholic/phenolic/alkane/alkene/alkyne/amine/ammonium/carboxylic group or halogen. All five isolated compounds (namely Ep-1 to Ep-5) exhibited mild to moderate irritant responses on the rabbit's skin. Maximum response was demonstrated by Ep-2 and Ep-4 when a dose of 120 µl of 1 mg/ml was applied on rabbit's ear. Reddening produced by these two compounds spread in an area of 3.0 cm. The irritant response of these compounds started after 1 h and reached to a +++ intensity after three hours in case of Ep-2 and four hours in case of Ep-4. The intensity of the reaction starts declining after about 8 h. The reaction lasted for about 72 h, and then clears. The compound Ep-1, Ep-3 and Ep-5, demonstrated a mild response, the reddening produced by these compound was spread in an area of 1.5 cm. It was further postulated from the results that the compounds Ep-2 and Ep-4 penetrated through the skin of rabbit's ear more simply as compared to other compounds. The presence of -OH, -COOH, or ketonic group and a double bond with conjugated diene system was accountable to react with the cell membrane and cellular ingredients of both the superficial and deeper layers of epidermis. As a result, inflammation of both the superficial and the deeper layers occurred, which probably hurt the epidermis. The mechanism of action of these compounds was possibly same as that of moderate to strong irritant compounds previously investigated by other scientists (Anderson et al., 1987; Benazra et al., 1985). There might be two possible reasons for the mild to moderate irritancy of +++ to + intensity displayed by Ep-1 to Ep-5. Initially, the compound itself penetrated to the skin. Secondly, the nature of the molecule was not so strong or severe enough to cause any terrible damage to the epidermal tissues of the skin but was strong enough to produce little dilatation of blood vessels and inflammation of the superficial layers.

Conclusion

Based on the data generated in this study, it could be concluded that a detailed chemical characterization of these phytochemical compounds is necessary, so that a structure-activity relationship of such important molecules in terms of irritant activity could be established.

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Full Length Research Paper

Evaluation of wound healing activity of the polyherbal and *Euphorbia hirta* formulations

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Evaluation of wound healing activity of polyherbal and *Euphorbia hirta* formulations was done. The study aimed at preparing a polyherbal formulation containing equal proportions of ethanolic extract of *Tridax procumbens*, *Euphorbia hirta*, *Eclipta alba*, dried rhizome of *Curcuma longa* and gel of *Aloe barbadensis*. Excision wound, incision wound and granuloma wound models were studied. In excision wound model it was observed that 100% wound healing was found on 12th day of the experiment on application of 10% polyherbal ointment and 100% wound contraction was found on 16th day of application of 10% ointment of *E. hirta*. The results were statistically compared with control and found significant. Similarly, period of epithelization, biochemical parameters and weight of granuloma tissues showed better results in the treated groups when compared with control in the studied wound models. Thus it is concluded that topical and oral formulations were found to contain significant wound healing activity.

Key words: Excision wound, incision wound, granuloma.

INTRODUCTION

More than 80% of the world's population still depends upon traditional medicines for various skin diseases (Babu et al., 2002). Herbal medicines in wound management involve disinfection, debridement and providing a moist environment to encourage the establishment of the suitable environment for natural healing process (Purna and Babu, 2000). Healing is the body response to injury in an attempt to restore normal structure and function. The process of healing involves two distinct processes: regeneration when healing takes place by proliferation of parenchymal cells and usually results in complete restoration of the original cells, and repair when that healing takes place by proliferation of connective tissue elements resulting in fibrosis and scarring (Harshmohan, 2002). Wound healing involves a

complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix. The phases of normal wound healing include hemostasis, inflammation, proliferation, and remodeling. Each phase of wound healing is distinct, although the wound healing process is continuous, with each phase overlapping the next. Open wounds are colonized by bacteria and systemic antibiotics are only indicated if invasive infections is present (Lorenz et al., 2003). The formulation which claims to possess wound healing activity shall be studied with the features like tensile strength of newly formed tissue, biochemical parameters like serum and tissue levels of hydroxyproline, lysyl oxidase, methionine, ascorbic acid etc. which contribute to the all round healing of wound.

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MATERIAL AND METHODS

Plants used

Gel of *Aloe barbadensis*, family Liliaceae containing glucomannan (Rastogi and Mehrotra, 1999), rhizomes of *Curcuma longa* family Zingiberaceae containing yellow matter curcumins (API, 1998), whole plant of *Eclipta alba* family Compositae containing coumestan derivatives, wedelolactones and demethyl-wedelolactone (Govindachari et al., 1956; Wagner et al., 1986), whole plant of *Euphorbia hirta* containing leucocyanidol, quercitol, camphol, quercitin and quercitol derivatives containing rhamnose and chlorophenolic acid (Rastogi and Mehrotra, 1999) and whole plant of *Tridax procumbens* containing β -amyrin, fucosterol and sitosterol, arachidic, behenic, lauric, linoleic, linolenic, myristic, palmitic and stearic acids (Rastogi and Mehrotra, 1999).

Extraction

Dried ethanolic extract of the dried rhizome of *Curcuma longa* and dried whole plants of *T. procumbens*, *Eclipta alba*, *E. hirta* was taken and gel of *Aloe barbadensis* was collected.

Formulation of topical and oral formulations

Topical formulation (ointment)

Two ointments were prepared by fusion method that is, one containing ethanolic extract of whole plant of *Euphorbia hirta* (10% w/w), and other containing equal proportions (2 g each) of ethanolic extract of whole plant of *T. procumbens*, ethanolic extract of whole plant of *E. alba*, ethanolic extract of whole plant of *E. hirta*, ethanolic extract of dried rhizomes of *Curcuma longa* and gel of *Aloe barbadensis* (10% w/w) in hydrophilic ointment USP base.

Oral formulation (suspension)

Two suspensions were formulated that is, one containing ethanolic extract of whole plant of *E. hirta* (10% w/v) and other containing equal proportions (2.5 g each) of ethanolic extract of whole plant of *T. procumbens*, ethanolic extract of whole plant of *E. alba*, ethanolic extract of whole plant of *E. hirta* and ethanolic extract of dried rhizomes of *C. longa* to make 10% w/v of suspensions.

Evaluation of *E. hirta* and polyherbal formulations for wound healing activity

All the animal studies were performed in Department of pharmacy, Barkatullah university, Bhopal. The department is an Animal Ethical Committee approved institution. The approval was granted by meeting held on 26/12/2006 with ref. No. BUPH/AEC/7865. The department is also approved for animal experimentation (Letter No. 444/01/C/CPCSEA Dated July 2001).

Excision wound model

For the excision wound study, each group containing six animals were selected. A wound of about 1 cm² area was made on the depilated dorsal thoracic region of rats. To make the wound, the rats were anaesthetized under light ether. The aseptic conditions were maintained and observed throughout the study. The wounded rats were individually caged and percentage wound closures were

observed from 2nd post wounding day to 22nd day, alternately (Morton and Malone, 1972).

Topical application

Group I (control)

The hydrophilic ointment base was applied topically, once daily.

Group II (Test 1)

10% ointment, in hydrophilic base, of 95% ethanolic extract of whole plant of *Euphorbia hirta* was topically applied, once daily.

Group III (Test 2)

10% polyherbal formulation was topically applied, once daily.

Oral administration

Group I (control)

2% tragacanth suspension was given orally, once daily, 200 mg/kg BW.

Group II (test 1)

10% w/v suspension of 95% ethanolic extract of whole plant of *E. hirta* in 2% tragacanth was given orally, once daily, 200 mg/kg BW.

Group III (test 2)

10% w/v suspension of polyherbal formulation in 2% tragacanth was given orally, once daily, 200 mg/kg BW. Parameter of percentage wound contraction and period of epithelialization were evaluated. Biochemical parameters like determination of hydroxyl-proline (Newman and Logman, 1950), hexosamine (Dische and Borenfreund, 1950) and protein content (Lowry et al., 1951) were also evaluated on 11th post wounding day. The percentage wound contraction was determined using the formula:

$$\% \text{ wound contraction} = (\text{Healed area} / \text{Total area}) \times 100$$

Wound contraction was measured each day using graph paper planimetrically.

Incision wound model

Thirty six male albino rats of either sex weighing 150 to 200 g were taken. Paravertebral incisions of 3 cm were made on the depilated skin of the rats anaesthetized under light ether with the help of a surgical blade. The incisions were sutured using 4 to 0 silk thread with cutting edges. Sutures were removed on the 8th post wounding day and the tensile strength were determined on 11th post wounding day by continuous constant water flow technique of Lee (Lee, 1968) of the rats of each group. White blood cell (WBC) count was also done to evaluate the extent of protection (Ehrlich and Hunt, 1969).

Table 1. Percentage wound contraction in excision wound model by topical application.

Days	Control	Test 1	Test 2
2	19.23± 0.94	25.41±1.05*	28.25±1.11*
4	22.31±0.87	42.83±0.90*	47.15±0.92*
6	27.7± 1.28	73.61±0.89*	75.72±0.96*
8	33.34±0.84	81.26±0.99*	92.36±1.07*
10	35.62±0.96	88.42±1.20*	97.42±0.95*
12	40.57±1.21	92.33±0.94*	100± 1.03*
14	44.12±1.17	96.14±1.13*	-
16	49.68±1.16	100±1.08*	-
18	53.21±0.91	-	-
20	57.81±1.10	-	-
22	60.33±0.88	-	-

Each group contain six animals, Mean ± SD. *Indicates significant activity at $p < 0.001$. #Indicates post wounding days.

Topical application

Group I (control)

The hydrophilic ointment base was applied topically, once daily.

Group II (Test 1)

10% ointment, in hydrophilic base, of 95% ethanolic extract of whole plant of *E. hirta* was topically applied, once daily.

Group III (Test 2)

10% polyherbal formulation was topically applied, once daily

Oral administration

Group I (Control)

2% tragacanth suspension was given orally, once daily, 200 mg/kg BW.

Group II (Test 1)

10% w/v suspension of 95% ethanolic extract of whole plant of *Euphorbia hirta* in 2% tragacanth was given orally, once daily, 200 mg/kg BW.

Group III (Test 2)

10% w/v suspension of polyherbal formulation in 2% tragacanth was given orally, once daily, 200 mg/kg BW. Measurement of tensile strength and total leucocytes count WBC (Shanbhag et al., 2005).

Dead space wound model

Thirty six albino rats of either sex were taken. Under light ether

anesthesia, subcutaneous dead space wound was inflicted in the paravertebral lumbar region by making a pouch through a small nick in the skin. Sterile polypropylene tubes measuring 2.5 cm in length and 0.5 cm in diameter were introduced in the pouch to induce granuloma formation. Each animal received two polypropylene tubes in the groin region. The wounds were sutured and mopped with an alcoholic swab. The harvesting of the granuloma tissue from the surrounding tissue was carried out on the 11th post wounding day. Then it was subjected to measurement of bearing strength of granuloma tissue and histopathological examination. The weight of wet and dry granulation tissue was measured along with the estimation of biochemical parameters like hydroxyproline, hexosamine and protein content (Turner, 1965).

Topical application

Group I (control)

The hydrophilic ointment base was applied topically, once daily.

Group II (test 1)

10% ointment, in hydrophilic base, of 95% ethanolic extract of whole plant of *E. hirta* was topically applied, once daily.

Group III (test 2)

10% polyherbal formulation was topically applied, once daily.

Oral administration

Group I (control)

2% tragacanth suspension was given orally, once daily, 200 mg/kg BW.

Group II (test 1)

10% w/v suspension of 95% ethanolic extract of whole plant of *E. hirta* in 2% tragacanth was given orally, once daily, 200 mg/kg BW.

Group III (test 2)

10% w/v suspension of polyherbal formulation in 2% tragacanth was given orally, once daily, 200 mg/kg BW.

DISCUSSION

The wound healing activity was evaluated by excision wound model, incision wound model, dead space wound model, burn wound model and induced diabetes wound healing model. In excision wound model, it was observed that 100% wound healing was found on 12th day of the experiment on application of 10% polyherbal ointment and 100% wound contraction was found on 16th day of application of 10% ointment of *E. hirta*. The results were statistically compared with control and found significant (Tables 1 and 2). On oral administration of the formulated

Table 2. Percentage wound contraction in excision wound model by oral administration.

Days [#]	Control	Test 1	Test 2
2	16.20 ±0.81	23.42±0.93*	38.11±1.26*
4	20.31±1.15	47.32±1.17*	49.66±0.99*
6	30.61±0.54	55.64±0.96*	58.15±0.93*
8	35.57±0.97	69.91±1.27*	66.62±1.36*
10	37.17±0.56	76.37±0.86*	79.41±1.26*
12	41.88±0.87	81.2±0.76*	85.0±1.42*
14	46.20±0.41	87.5±0.88*	100±0.93*
16	49.42±0.98	94.41±1.20*	-
18	55.90±1.15	100±1.31*	-
20	59.50 ± 0.87	-	-
22	62.76±0.84	-	-

Each group contain six animals, Mean ± SD; [#]Indicates Post Wounding Days *Indicates significant activity at P < 0.001.

Table 3. Period of epithelization (days) in excision wound model.

Groups	Period of epithelization (days)	
	Topical (ointment)	Oral (suspension)
Control	26.17±1.15	28.45±1.21
Test 1	15.12±0.85	17.20±1.22
Test 2	11.61±1.10	13.12±1.34

Each group contain six animals, Mean ± SD. *Indicates significant activity at P < 0.001.

Table 4. Biochemical Parameters of Excision wound model on Topical Application on 11th Post Wounding Day.

Parameter (mg/g tissue wt.)	Control	Test 1	Test 2
Hydroxyproline	19.1±1.3	31.3±2.3*	74.1±2.5*
Hexosamine	3.3±0.4	12.1±1.3*	24.3±2.0*
Protein	19.6±1.3	35.0±1.8*	68.3±1.0*

Values are expressed as Mean±SD * indicates significant activity P < 0.01.

Table 5. Biochemical parameters of excision wound model on oral administration on 11th post wounding day.

Parameter (mg/g tissue wt.)	Control	Test 1	Test 2
Hydroxyproline	16.8±2.1	28.4±1.8*	59.5±2.2*
Hexosamine	5.1±0.2	9.5±1.1*	20.2±0.23*
Protein	17.3±1.2	31.3±1.0*	56.0±1.8*

Values are expressed as Mean±SD; *Indicates significant activity P < 0.01.

suspensions it was found that 100% wound contraction on 14th day was shown by rats treated with polyherbal suspensions whereas complete wound contraction was found on 18th day in *E. hirta* treated group. The results were compared statistically with control and found satisfactory (Table 3). The period of Epithelization was also found to be significantly shorter for the treated groups as compared to control. However the results were more encouraging for the polyherbal ointment (Table 4). Estimation of biochemical parameters- hydroxyproline, hexosamine and protein content was done on the 11th post wounding day and it was found that the respective concentrations of the parameters increased in the treated groups, however the increase was most marked in the topically applied polyherbal formulation followed by polyherbal oral formulation. It was reported that besides collagen C1q of the complement system contains considerable amount of collagen (Nagelschmidt and Struck, 1977). The increase in the biochemical parameters of the topical and oral *E. hirta* formulations was also found to be significant when compared to control (Tables 5 and 6). The period of epithelization was found to be least in the topical polyherbal formulation and that of the oral polyherbal was found to be better than *E. hirta* topical formulation.

In the incision wound model, the tensile strength of the new generated tissue was measured. Collagen, the major protein of the extracellular matrix, is the component that ultimately liberates free hydroxyproline and its peptides (Nayak and Pereira, 2006). The tensile strength of a wound is determined by the rate of collagen synthesis and more so, by the maturation process where there is a covalent binding of collagen fibrils through inter and intra molecular cross linking (Malviya and Jain, 2009). It was found that the tensile strength of new tissue was more in the treated groups with least significant signs of infection. The strength of the tissues was statistically significant in the polyherbal topical group followed by polyherbal oral group (Table 6).

In dead space wound model, it was found that the weight of wet and dry granuloma tissue formed was more in the case of treated groups when compared to control. The increase in the weight of the granuloma was most significant in the topically applied polyherbal formulation groups followed by orally administered polyherbal groups. Similarly, the rise in the biochemical parameters like those of hydroxyproline, hexosamine and protein content of the granuloma tissue was lot more in both topically and orally treated groups. However, the results were most encouraging and statistically significant in the topically treated polyherbal formulations, when compared with control (Table 7 and 8). Histopathological studies showed formation of scanty collagen tissue in the control group (Figures 1 and 2). The test groups treated with polyherbal topical formulations showed formation of dense collagen tissues with minimum presence of macrophages (Figure 2d) and formation of complete epidermis (Figure 2c).

Table 6. Effect of formulations on tensile strength and WBC count in incision wound model.

Group	Tensile strength (g)		WBC count ($\times 10^3$ cell/mm ³)	
	Topical	Oral	0th Day	10th Day
Control	223 \pm 3.5	215 \pm 3.3	13.95 \pm 0.51	30.52 \pm 1.15
Test 1	330 \pm 4.2*	302 \pm 3.3*	14.0 \pm 0.62*	13.98 \pm 1.10*
Test 2	360 \pm 6.3*	347 \pm 4.1*	14.0 \pm 0.46*	13.97 \pm 1.12*

Table 7. Physical and biochemical parameters by topical application in dead space wound model.

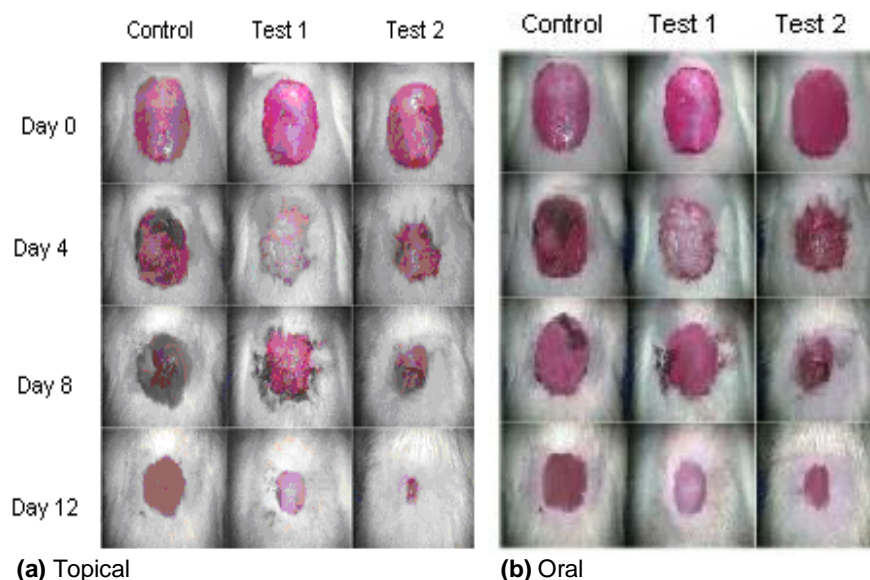
Parameter	Control	Test 1	Test 2
Wet granulation weight (mg/100 g)	229 \pm 11.2	380.41 \pm 13.2*	483.4 \pm 15.3*
Dry granulation weight (mg/100 g)	25.1 \pm 3.1	77.0 \pm 1.4*	94.3 \pm 1.5*
Protein (mg g ⁻¹)	19.71 \pm 1.5	34.1 \pm 2.1*	64.1 \pm 2.1*
Hydroxyproline (mg g ⁻¹)	16.93 \pm 1.2	22.4 \pm 2.6*	30.9 \pm 2.1*
Hexosamine (mg g ⁻¹)	9.74 \pm 0.05	16.4 \pm 1.9*	28.1 \pm 1.4*

All values are expressed as mean \pm S.D. *P < 0.01 significant compared to control.

Table 8. Physical and biochemical parameters by oral administration in dead space wound model.

Parameter	Control	Test 1	Test 2
Wet granulation Weight (mg/100 g)	220 \pm 10.4	289.5 \pm 14.1*	415.2 \pm 10.3*
Dry granulation weight (mg/100 g)	31.3 \pm 1.6	70.0 \pm 1.2*	89.1 \pm 2.3*
Protein (mg g ⁻¹)	21.5 \pm 1.4	50.5 \pm 2.1*	62.1 \pm 1.5*
Hydroxyproline (mg g ⁻¹)	3.2 \pm 0.08	5.12 \pm 1.5*	24.96 \pm 1.3*
Hexosamine (mg g ⁻¹)	8.5 \pm 0.05	14.8 \pm 1.2*	23.6 \pm 1.5*

All values are expressed as mean \pm SD. *P < 0.01 significant compared to control.

**Figure 1.** Wound contraction in excision wound model.

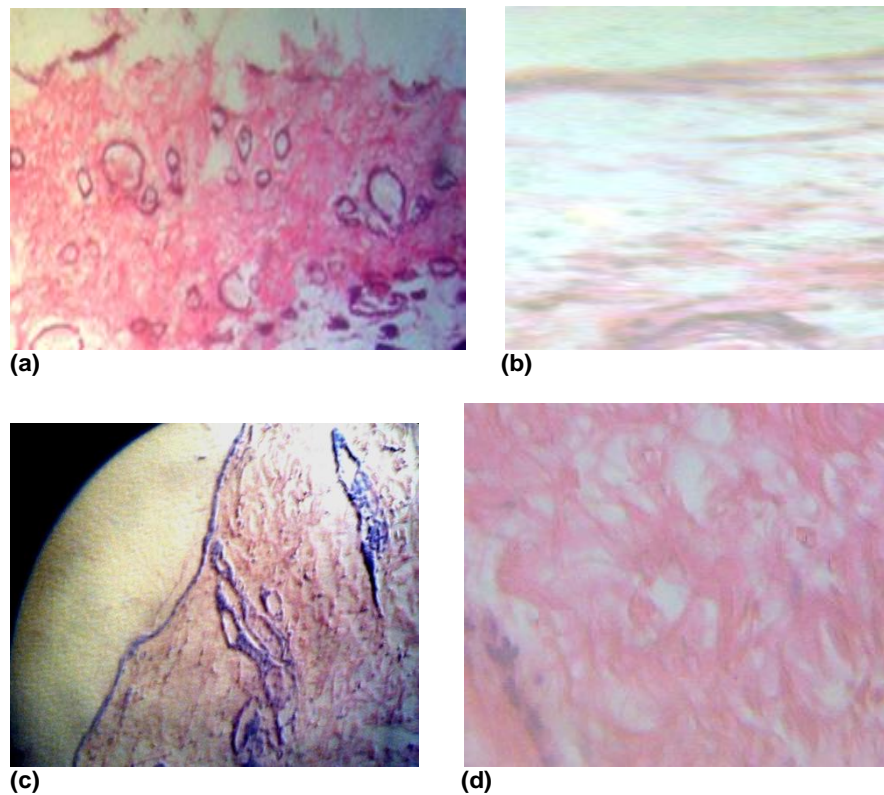


Figure 2. Histopathology of dead space wound model (a) control (oral), (b) control (topical), (c) Test 1 (topical), (d) Test 2 (topical).

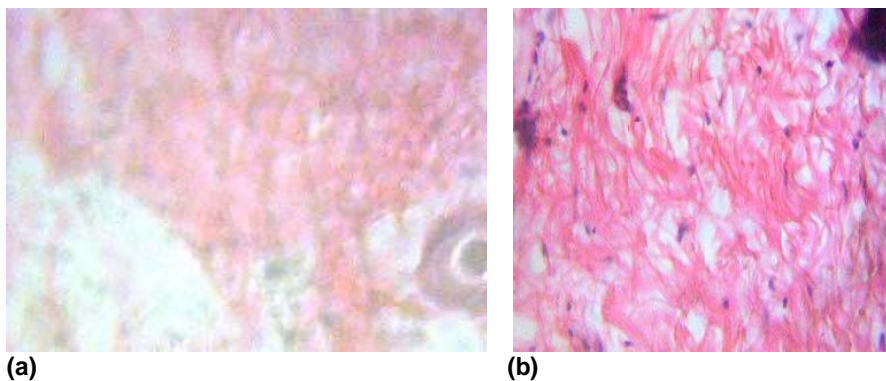


Figure 3. Complete epidermis (a) Test 1 (oral), Test 2 (oral).

The best groups treated with polyherbal oral formulations also showed formation of dense collagen tissues, but slightly less than topically treated groups by polyherbal formulations (Figure 3b). The test group treated with topical formulation of *E. hirta* showed formation of collagen tissues with presence of macrophages (Figure 2c), whereas those treated with oral *E. hirta* formulations showed collagen tissues less than in the topically treated groups of *E. hirta* (Figure 3a).

The wound healing studies revealed that the polyherbal

and *E. hirta* formulations possess significant activity but it may be concluded that the polyherbal topical formulations has got remarkable results in all types of wound healing protocols (Figures 4 and 5). The study has concluded to acclaim *E. hirta* as a prominent wound healer (200 mg/kg BW).

Conclusion

The polyherbal formulations containing equal proportions

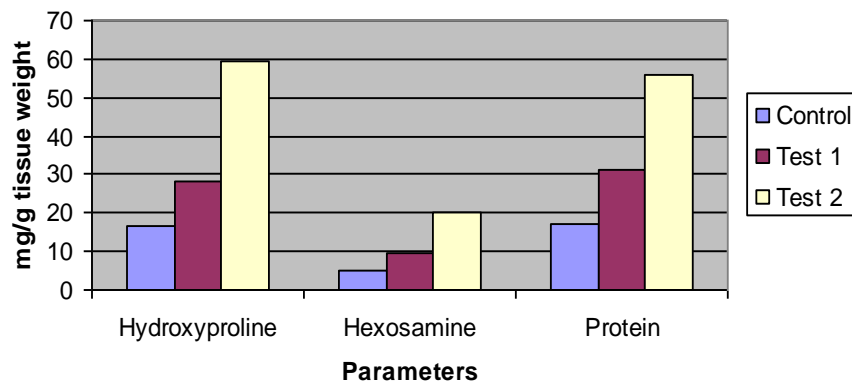


Figure 4. Biochemical parameters of excision wound model on oral administration

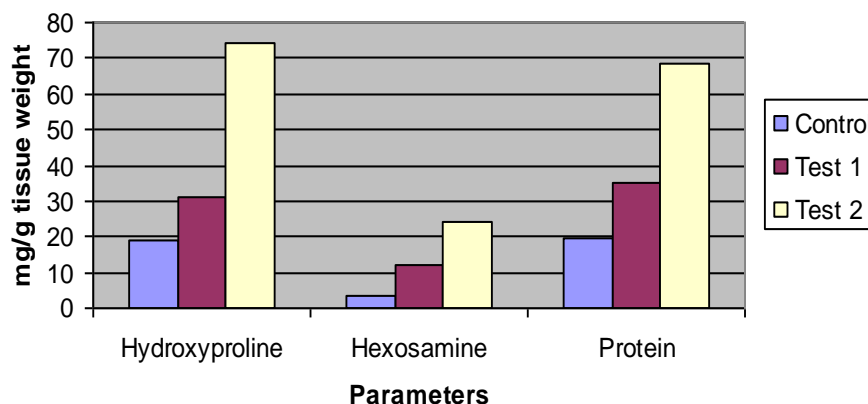


Figure 5. Biochemical parameters of excision wound model on tropical administration.

of the ethanolic extracts of the plants studied can be used both topically and orally for the healing of wounds. Single or multiple factors may play a role in any one or more individual phases, contributing to the overall outcome of the healing process (Guo and Di Pietro, 2010).

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Full Length Research Paper

Design, development, and evaluation of antimicrobial activity of herbal antiseptic wound pad-neemplast

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There is growing demand of polyherbal formulations in the world market. The present study aimed to design, develop, and evaluate the antiseptic activity of herbal wound pad Neemplast containing distillate of Neem leaves (*Azadirachta indica*) and Black pepper (*Piper nigrum*) and the oil of clove (*Syzygium aromaticum*) and Eucalyptus (*Eucalyptus globules*). The plants have been reported in the literature as having good antimicrobial, anti-oxidant and anti-inflammatory activity. Various formulation batches were prepared and evaluated for various parameters like colour, appearance, pH, weight per ml, assay identification and antimicrobial activity. The formulation of Batch# 358-K-12 was compared with the marketed preparation Sani-plast. It is a very good attempt to establish the herbal antiseptic wound pad containing distillate of Neem leaves Black pepper. The plant trials were conducted on a commercial scale machines in order to observe behavior and feasibility of machine with respect to new product formulation and also initiated analytical studies (qualitative and microbiological determination) in order to get the physical and chemical compatibility of formulation with the wound pad material. Implement current good manufacturing practice (cGMP) concept during all the manufacturing and packaging process. Neemplast wound pad was successfully designed and developed after extensive manufacturing and evaluation process by specialized techniques for evaluation of antiseptic activity *in vitro*.

Key words: Antiseptic, distillate, *Piper nigrum*, *Azadirachta indica*.

INTRODUCTION

Neemplast wound pad consist of sterilized non-woven (70% viscose 30% polyester) antiseptic pad impregnated with Neem leaves (*Azadirachta indica*) and Black pepper (*Piper nigrum*) distillate along with Clove (*Syzygium aromaticum*) and Eucalyptus oil (*Eucalyptus globules*) which is covered by releasing and printed paper (Wink and Wyk, 2004; PDR, 2004). It is used to protect and heal cuts, scratches, blisters, insect bites and minor wounds. It is new antiseptic polyester fabric adhesive bandages as the elementary material is more suitable to

be used for children as well as for adults. For its more advantages, each bandage is wrapped individually in a waterproof bag. The outer package is strong cardboard carton to keep best storing condition (Monteiro-Riviere et al., 2005). Neem leaves show pharmacological actions as, analgesic, antibacterial, antifungal, and anti-inflammatory properties (Parotta, 2001; Ross, 2001). Clove oil possesses antimicrobial potential (Dorman and Deans, 2000; Betoni et al., 2006). Eucalyptus oil has anti-inflammatory, analgesic and antimicrobial activities used

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Table 1. Neemplast specifications for bulk solution and finished product (strip).

Parameter	Norm
Appearance	Light yellow to colorless transparent liquid with characteristic odor
pH	4.5 – 6.5
Weight per ml	(0.967 – 1.070) g/ml
Assay Identification: Eugenol	Positive
Finished product (bandages)	
Description	Transparent P.E film ventilated bandage pad impregnated with Neem and black pepper distillate along with clove and eucalyptus oil which is covered by releasing paper and printed paper
Identification	Eugenol
Adhesive skin test	Easily removes from skin without any adhesive deposition.
Average weight (g)	0.5226±5% (0.494 –0.546)
Bandage length (mm)	72±1
Bandage width (mm)	19±1

traditionally (Cimanga et al., 2000; Benayache et al., 2001; Boland and Brophy, 1991). Black piper (*Piper nigrum*) has antimicrobial activity and is used as antibacterial against different microorganisms (Dorman and Deans, 2000; Perez and Nesini, 1994). Its designed antiseptic polyester fabric adhesive bandages as the elementary material is more suitable to be used for children as well as for adults. Each bandage is wrapped individually in a waterproof bag. The outer package is strong cardboard carton to keep best storing condition. The idea for designing the Neemplast was taken from the Shanghai Huazhou PSA Products Co., Ltd. This prospective study was to be conducted to define and prove that the Neemplast purported function in a consistent and reproducible manner as per manufacturing process. Neemplast is manufactured by combination of medicinal plants and there is no synthetic material used in manufacturing of Neemplast wound pad. This type of innovation is alternate to synthetic wound pad Sani-plast with almost similar efficacy and antiseptic activity. *A. indica* and *P. nigrum* are reported to possess very beneficial effect on skin due to their anti-microbial, anti-inflammatory and anti-oxidant activities.

MATERIALS AND METHODS

Collection of plant material

Leaves of neem and black pepper were purchased from the local market Jooria bazaar, Karachi while clove oil and eucalyptus oil were collected from the Supply Chain Department of Herbion Pakistan Private Limited.

Preparation of extracts

1.25 kg of dry Neem leaves was taken and properly washed. 8.75 liters of D.I water in extractor was collected and herb placed in it. This was stirred and heating till boiling. The temperature was from 110-120°C. Then temperature was reduced and maintained up to

90-100°C for 30 min. When extraction was completed, the steam was release and the aqueous extract filtered through mesh number 100. After filtration, the filtrate was transferred to evaporator. The filtrate was concentrated through evaporation. The temperature was from 100-110°C. 1.25 gm of methyl paraben and 0.25 gm of propyl paraben was added to the concentrated thick extract and stirred for 10 min, and the desired concentration of extract was obtained.

Development of formulations

Different batches were prepared according to the Table 1. The desired concentration of extract, oils and emulsifying agent were weighed accurately and following these parameters light viscous and smooth texture that gave shiny, soft surface with smooth impregnation, low squeezer gauge 4.4 mm for more retention of active on non impregnated wound roll and kept low dryer temperature 170 -172°C that role in prevention from over drying and degradation of actives and reduced stress and load on dryer heater, high speed F 40.45 Hz for maximum output of product. Prepared the solution and finally dipped the wound pad roll into the bulk solution. The specification of bulk and finished product is shown in Table 1. The manufacturing of herbal antiseptic neem plast wound pad involves the following stages: Preparation of neemplast solution and impregnation of wound pad

Preparation of Neemplast solution

First step in manufacturing of Neemplast is preparation of Neemplast solution which involves preparation of two phases separately and then mixing them together to get the final solution.

Phase I (water phase): Took accurate amount or quantity of Neem and Black pepper distillate previously obtained from Neem leaves (that is, 500 gm) and black pepper fruit (12.5 gm) in stainless steel vessels. Then add Benzalkonium chloride and mixed well with constant stirring for 10 min. Added and dissolved EDTA to got clear, transparent solution.

Phase II (oil phase): Took Eucalyptus and clove oil in a beaker and mixed well. Added hydrogenated castor oil and polysorbate 20 and mixed with continuous stirring. Then transferred gently Phase II (Oil phase) into Phase I (water phase) with constant stirring to got homogenous uniform and clear solution. Make up volume with distilled water and mixed well. Then checked the pH of solution that was in range of 5.0- 6.0.

Table 2. Parameters for neemplast assessment.

S/N	Parameter	Norm
1	Description	Transparent P.E film ventilated bandage pad impregnated with Neem, Black pepper distillate along with Clove and Eucalyptus oil which is covered by releasing and printed paper.
2	Identification	Eugenol (must be positive)
3	Adhesive skin test	Easily removes from the skin without any adhesive deposition.
4	Average weight of strip with releasing and packing paper (g)	0.5226±5% (0.495 – 0.546)
5	Average weight of strip with releasing paper only (g)	0.236 (0.220 – 0.300)
6	Wound pad length (only) (mm)	24 ±1
7	Wound pad width (only) (mm)	13±1
8	Bandage Length (without releasing and packing paper)	72±1
9	Bandage Width (without releasing and packing paper)	19±1

Impregnation of wound pad

After getting solution, next step was to impregnate the wound pad with it, which involves following stages.

Impregnation machine process and behavior

Rim/ wound Pad roll: 6 non-impregnated rolls/ rims easily installed on Impregnation machine rollers and easily connected by stretching and fixing them on the other side of rollers. Switched on impregnation machine and adjusted the machine as per below specification: (Air flow control: 3 inches, squeezer gauge: 5 mm, speed: F40.45 Hz and thermostat temperature: 170 to 180°C).

Product (solution) holding pocket/ duct filled easily neemplast solution in the pocket deep up to desired level. Filled the Neemplast herbal solution in the solution duct and started the impregnation process, the wound pad rollers started moving and dipping or soaking into the solution by aid of a moving arm fix at the top of solution duct. After dipping and squeezing the wound pad reaches in to drying chamber for drying. End or receiving roller were moving well and receiving the soaked, saturated and dried impregnate wound pads.

Medicated plaster shape cutting and packing machine process and behavior

Fixed the dried impregnated rolls, release paper, printed and unprinted paper packing rolls on plaster shape cutting and packaging machine and started the process. Plaster shaping was done smoothly by cutting wound pad roll according to the pre-defined specification.

Paper packing and positioning was done accurately in connection with plaster shaping. Sealing and cutting were done nicely by push- punch type cutter. Optical test were performed on finished strips under white florescent light and found all of them centrally aligned.

Quantitative and microbiological test conducted on bulk and finished products and found satisfactory results. Finished product (bandage) specifications and flow chart of the process by using impregnation and medicated plaster shape cutting and packing machine as shown in Table 2 and Figure 1.

Evaluation of formulations

Physical evaluation (appearance)

Light yellow to colorless transparent liquid with characteristic odor were checked visually.

pH

pH of aqueous solution of the formulation was measured by calibrated Hanna pH meter (HI 110).

Weight per ml

It was determined by pycnometer at the specified temperature (24°C).

Quantitative estimation of eugenol in neemplast by high performance thin layer chromatography (HPTLC)-densitometry

The quantity of Eugenol in Neemplast was observed by using HPTLC-Densitometry. In this process the equipment CAMAG Scanner III, CAMAG Linomate 5 or Equivalent and HPTLC silica gel G60F₂₅₄ were used. It also included solvent system named n-Hexane: Ethyl acetate: formic acid (8:2:0.1) with wave length: 254 nm. For this analysis, there were two steps involved as given below:

Standard preparation

Prepared standard solution by dissolving 10 mg Eugenol standard and 5 ml of methanol in 10 ml volumetric acid flask with continuously shaking. The volume was adjusted upto the mark with methanol.

Sample preparation

Weighed about 1.0 gm wound pad of Neemplast in 100 ml conical flask, added 10 ml of methanol quantitatively by using graduated pipette, covered it properly to avoid evaporation of solvent. Stirred it

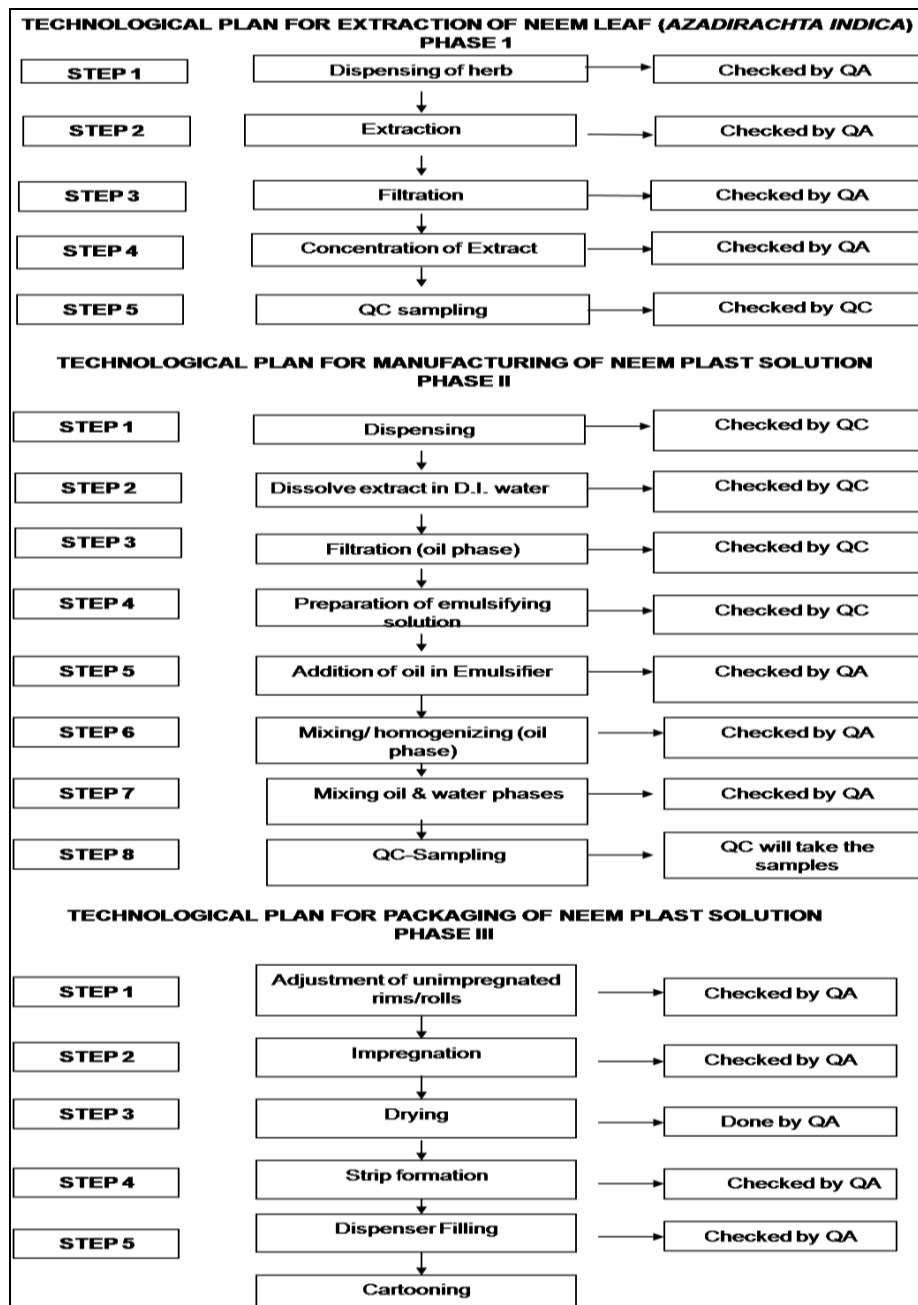


Figure 1. Flowchart of Neemplast development.

for 20 minutes, sucked the solvent through dropper or pipette and filtered the solution carefully through Whatman filter paper No. 44 and used filtrate as sample for sample application.

TLC preparation

Performed analysis on 10 x 10 cm HPTLC silica gel G60F₂₅₄ plates with fluorescent indicator. Before started the analysis, HPTLC plate was cleaned by predevelopment with the methanol by ascending method. (Note: Immerse HPTLC Plate in a CAMAG glass chamber (20 x 10 cm), contains 30 ml methanol (HPLC grade) as solvent system cover the chamber with glass lid and wait to develop the

plate to the top with methanol. After complete development, removed the plate from TLC glass chamber and dry it in an oven at 105°C for 5 min).

Application procedure

Applied three spots of 10 µl (in the form of band) of standard preparation along with three spots of 10 µl of sample preparation as the bands on the same plate by means of a CAMAG Linomat 5 (automated spray-on applicator equipped with a 100 µl syringe and operated with the settings band length 6 mm, distance between bands were 14 mm, distance from the plate side edge was 15 mm,

and distance from the bottom of the plate was 15 mm). After sample application dried the plate in hot air oven for 5 min at 105°C.

TLC development

Developed the plate by immersing sample HPTLC Plate in a CAMAG glass chamber (20 x 10 cm) contained the solvent system (n-Hexane: Ethyl acetate: Formic acid (8: 2: 0.1)), wait to develop the plate to a distance of 8 to 9 cm. After complete development, allowed the plate to dry by keeping in open air for one day.

TLC scanning

Scanned the plate next day in the densitometer by linear scanning at 254 nm by use of a TLC Scanner III CAMAG with a mercury lamp, and integrate the area of the spots corresponding to Eugenol standard as show in Figure 3. Calculated the amount of Eugenol in mg per capsule by following formula.

$$\text{Content of eugenol} = \frac{A_{\text{SMP}} \times W_{\text{STD}} \times \text{dilution of SMP} \times P \times W_{\text{AVG}}}{A_{\text{STD}} \times \text{dilution of STD} \times W_{\text{SMP}} \times 100}$$

Where, A_{SMP} , Avg. area of sample; A_{STD} , Avg. area of standard; W_{STD} , weight of standard, mg; W_{SMP} , weight of sample, g; P, percent purity of standard; W_{AVG} , average weight of neemplast wound pad.

Microbial assay

The antimicrobial activities of different formulations were determined by tryptic soy agar (TSA), tryptic soy broth (TSB), lactose and peptone water test tubes. In this method, stock American Type Culture Collection (ATCC) culture of *Bacillus subtilis* (British and Americal Pharmacopoea) was incubated in TSB for 3 -5 days. When growth occurred streak on Petri plates containing TSA media in order to get isolated colonies, incubated it at 32 ± 2.5 C for 1 day. After incubation period, took heavy loop full of cultures and inoculated it into test tube containing peptone water that was dedicated for that particular organism. Next step was to make serial dilution in peptone water test tubes from 1: 10 to 1:10⁸. Then took 1 ml from each tube and transferred it into Petri plate that was specifically mark for that particular organism. Then poured 15-20 ml TSA in each plate and incubated for 3 days. Kept all the test tubes in refrigerator. After incubation period read plates and counted number of colonies forming unit at each dilution factor. All results were recorded. For comparison of test sample (Neemplast) and control sample (Sani-plast) culture of *B. subtilis* from TSB agar poured into melted TSA agar that is, (40 - 45°C). The plates were allowed to solidify. Incubated all the plates for 24 h. The test was performed in duplicate. Next day took out all plates and placed Neemplast strip on Petri plate with the help of sterile for escape, after that incubated all these plates for 2 – 3 days. The antibacterial activities were observed by measuring the zone of inhibition (mm) of both the Neemplast and Sani-plast with the help of Vernier Caliper. Recorded the results and compared zone of inhibition of sample with standard (Sani-plast-Acrinol) as shown in Figure 2. On the basis of the observations we can conclude that the result was satisfactory. The prepared dosage form of Neemplast was evaluated for various evaluation parameters such as general appearance, and weight variation, antimicrobial activity. The final formulation found to have light yellow to colorless transparent liquid with characteristic odor and had pH 4.5-6.5 with average weight of 0.5226 g \pm 5% (0.494 to 0.546 g).

RESULTS AND DISCUSSION

Neemplast was evaluated in terms of appearance, and found light yellow to colorless transparent liquid with characteristic odor were checked visually. pH of aqueous solution of the formulation was also measured and weight per ml was determined by pycnometer and with the help of HPTLC-Densitometry the active (Eugenol) of Neemplast was determined. By analyzing antimicrobial activity it was found that newly developed herbal antiseptic wound pad Neemplast had an inhibitory effect on the *B. subtilis*. It also showed satisfactory zone of inhibition compared with control sample. Neemplast is based on natural source and showed zone of inhibition very close to Acrinol based Sani-plast, so we can conclude that Neemplast has similar efficacy and safe to use as compared to synthetic (Acrinol based) Sani-plast. In this way Neemplast solution and finished formulation showed comparatively satisfactory antimicrobial activity than control sample.

The present study shows that newly developed polyherbal anti septic wound pad was successfully designed, developed and assessed its activity by antimicrobial activity against control acrinol based Sani-plast. In addition, the quantity of active ingredients eugenol in Neemplast was determined by using HPTLC-Densitometry. Hence herbal wound pad could be used as better and safe substitution of synthetic wound pad Sani-plast. In previous studies it is described that when leaf of Neem are boiled in water, it serves as an excellent antiseptic to clean the wounds, soothes the skin, swellings and also eases skin problems (Shahidi et al., 2004). When applied against Gram-negative and Gram-positive microorganisms, the Neem oil from the leaves, seeds and bark have shown a wide spectrum of antibacterial activity (Chopra et al., 1956). It is reported that Neem oil has the antimicrobial activity against a variety of pathogens like *Vibrio cholerae*, *Klebsiella pneumoniae*, *M. tuberculosis* and *M. pyrogenes* in vitro study (Satyavati et al., 1976). The extract of Neem has shown antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae* and *B. subtilis* (Jagannadh and Radhika, 2006; Shravan et al., 2011). The leaves of Neem are locally applied on skin as poultice for curing boils, and also used in as antiseptic for healing and cure of wounds, ulcers and eczema. Traditionally it is said that Neem leaves are used to treat itching and other skin diseases, and even bathing with Neem leaves has shown beneficial effects to cure skin ailments (Ghani and Khazainul, 2004; Chatterjee and Pakrashi, 2011). Against *B. subtilis* methanol extract of *Azadirachta indica* showed marked activity (28 mm) (Shravan et al., 2011). Active ingredients of Neem works in wound healing process and also help the skin to retain its agility as the wound heals (Pandey et al., 2011). It contains terpenoids that helps wound healing (Hawkins and Ehrlich, 2006). Eucalyptis oil has wound healing properties mainly due to monoterpenes (Sarkar, 1994). Oil of eucalyptus has been

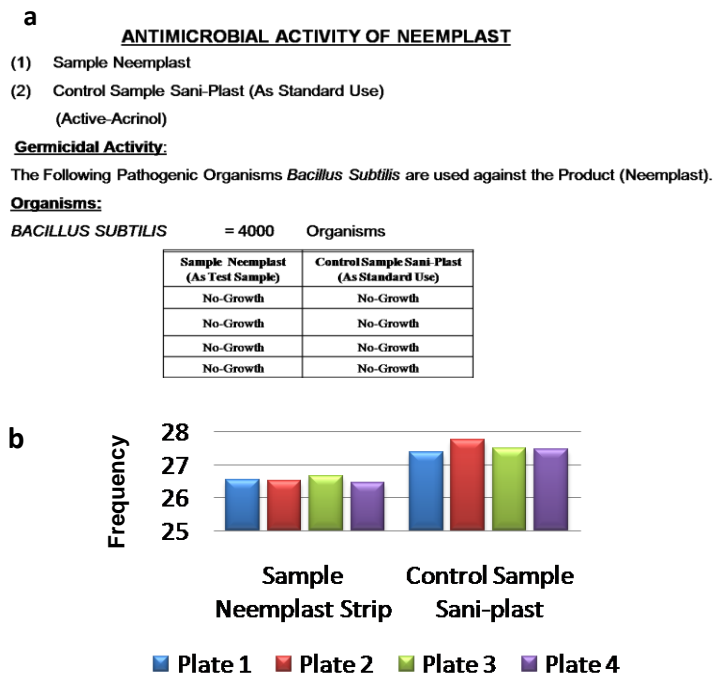


Figure 2. (a) Microbiology report No growth was observed for neemplast; (b) graphical representation of antimicrobial activity of test (Neemplast) and control (Sani-plast) sample.

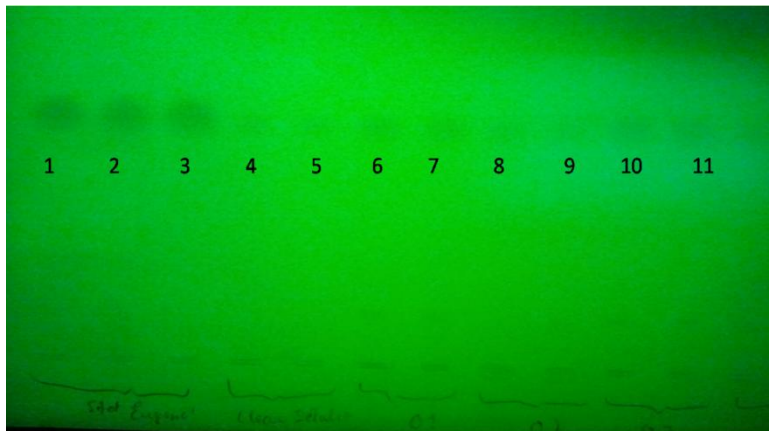


Figure 3. Quantitative estimation of eugenol in neemplast by HPTLC-densitometry. 1, 2 and 3, eugenol reference standard; 4 and 5, neem plast bulk solution; 6 and 7, wound pad 0; 8 and 9, wound pad 02; 10 and 11, wound pad 03.

traditionally used in Ayurveda as an antiseptic and also it has reported that this oil has antibacterial action (Nadkarni, 1979). Topical application is effective against different microorganisms and also reported antibacterial action (Sherry et al., 2001; Kumar, 1988; Ahmad and Beg, 2001). The prepared dosage form of Neemplast was evaluated for various evaluation parameters such as general appearance and weight variation, antimicrobial

activity and quantitative analysis. The prepared bandage consist of sterilized non-woven (70% viscose, 30% polyester) antiseptic pad impregnated with Neem and Black pepper (Patani, 2002) distillate along with Clove and Eucalyptus oil (Usmanghani et al., 2009) which is covered by releasing and printed paper. The final formulation found to have light yellow to colorless transparent liquid with characteristic odor and had pH 4.5

to 6.5 with average weight of $0.5226 \text{ g} \pm 5\%$ (0.494 to 0.546 g).

Conclusion

Herbal dosage forms of *Neemplast* showed good elegance and appearance. It is an excellent effort to design and develop the herbal antiseptic wound pad having satisfactory zone of inhibition and antimicrobial activity comparable with control sample Sani-plast. By using HPTLC-Densitometry Eugenol (*Neemplast*) was detected in comparison with Acrinol (*Sani-plast*). This study revealed that the developed herbal wound pad was suitable dosage form for antiseptic bandages.

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Full Length Research Paper

Comparison of the effect of azithromycin versus erythromycin on gallbladder motility: A sonographic study

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Erythromycin is a known prokinetic (cholecystokinetic) drug. Recently, erythromycin has been linked to the occurrence of arrhythmias and cardiac death due to QT prolongation. Azithromycin is similar to erythromycin in structure, but has the least arrhythmogenic tendency among all the macrolides. This study was aimed at determining the comparative cholecystokinetic effects of erythromycin and azithromycin. Twenty four apparently healthy males were studied in pre-prandial and postprandial states. Thirty minutes before the study (after an overnight fast), the subjects took 500 mg azithromycin and erythromycin in a randomized cross over method. Immediately before the ingestion of a standardized liquid meal, the length, width and height of the gallbladder was measured in each subject to obtain the ellipsoid volume using real time sonography and in supine position. After the ingestion of the liquid meal, the gallbladder measurements were obtained every 5 min for 40 min. The gallbladder contraction index (GBCI) was calculated for each period as a percentage change in volume using the fasting volume as the initial volume in all the calculations. The weight, height and age of each subject were obtained. Statistical analysis was conducted using Statistical Package for Social Sciences (SPSS) software; paired *t*-tests were used to compare GBCI values in erythromycin and azithromycin interventions. $P < 0.05$ was the criterion for statistical significance. In majority of the periods, erythromycin showed significantly higher GBCI values than azithromycin; azithromycin showed higher GBCI values in few points. Erythromycin has cholecystokinetic superiority over azithromycin. From tolerance point of view, azithromycin should be the preferred drug as it does not have significant drug-drug interaction and may be a potential new treatment of cholestasis.

Key words: Sonography, erythromycin, azithromycin, gallbladder motility, cholecystokinesis.

INTRODUCTION

Gallbladder motility can be affected by various clinical conditions such as obesity, diabetes mellitus and celiac disease. In obesity, a condition characterized by

increased risk of gallstone(s) as a result of decreased gallbladder motility has been reported (Fraquelli et al., 2003). Furthermore, decreased gallbladder motility are

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contributed by other factors including autonomic neuropathy, which reduces gallbladder sensitivity to cholecystokinin (CCK) or reduced the numbers of CCK receptors on gallbladder wall. CCK is not easily accessible in the therapy for gallbladder stasis, so this is an alternative. Progesterone (P) has an inhibitory effect on the contractility of gastrointestinal smooth muscle, including the gallbladder. Since P levels are elevated during pregnancy, biliary stasis may develop during pregnancy. This is characterized by an increase in fasting and residual volumes, and also by a decrease in emptying capacity (Kline et al., 2005).

Impaired gallbladder motility and delayed intestinal transit contribute to cholesterol and gallstone formation by impeding the enterohepatic circulation of bile salts and causing gallbladder stasis (Xu et al., 1998). The therapeutic value of erythromycin, a prokinetic motilin analogue, was evaluated in an animal model for gallstone formation. Erythromycin treatment for animals on high cholesterol diet, restored gallbladder contractility and intestinal transit to control levels, increased bile salt secretion, reduced the total bile salt pool, lowered the cholesterol saturation of bile, lengthened the nucleation time, and also reduced crystal formation. Erythromycin enhances gallbladder motility and hastens intestinal transit thereby promoting rapid enterohepatic cycling of bile salts. This increased bile secretion improves cholesterol solubility and reduces crystal development (Xu et al., 1998). Furthermore, erythromycin has been shown to be better than other commonly used prokinetic agents like domperidone, metoclopramide and cisapride as tachyphylaxis, and other adverse effects are obstacles to their use (Frazee et al., 1994).

Several reports of arrhythmias associated with use of either oral or intravenous (IV) erythromycin have been reported (Ray et al., 2004; Wisialowski et al., 2006; Milbery et al., 2002). In 2004, a large cohort study of Tennessee Medicaid patients from 1988 to 1993 showed a possible association between erythromycin with a risk of cardiac death through its extensive metabolism by the cytochrome P-450 3A isoenzymes (Ray et al., 2004). In his study, the adjusted rate of sudden death from cardiac causes was five times higher in patients on erythromycin than in patients on other antibiotics such as amoxicillin.

A previous study on the comparative prokinetic effect of erythromycin and azithromycin was centred on gastric motility (Moshiiree et al., 2010). There has not been any sonographic study to the best of our knowledge, on the comparative cholecystokinetic effect of erythromycin and azithromycin using randomized controlled data. As a result, to the aim of this work was to study the comparative effect of erythromycin and azithromycin on gallbladder motility in apparently healthy subjects.

MATERIALS AND METHODS

This study was a single-centre, randomized, single-dose two way cross-over study in apparently healthy adult male volunteers. This

study was placebo-controlled so as to avoid confounding variables like subject differences e.g. weight which will affect the validity of the research. The study was conducted in Leeds Hospital, Anambra State, Nigeria. In line with Helsinki Declaration, approval for this study was obtained from the Human Research Ethics committee of the Nnamdi Azikiwe University, Nnewi Campus, Anambra State. The procedures were explained to the subjects (volunteers) and each subject signed consent form before enrolling into the study. All subjects were aware of their option to withdraw from the study anytime they desired.

This study involved 24 apparently healthy male volunteers. This number was considered to have sufficient power based on prior experiences in studies with a similar design (Schauch et al., 1988; Mannaerts et al., 1998). Potential study subjects underwent medical history, fasting blood sugar tests, and physical examinations. Exclusion criteria included history of hepatobiliary diseases, gastrointestinal or metabolic disease (e.g. diabetes). Subjects who had chronic diseases (diabetes, sprue, achalasia, irritable bowel syndrome, truncal vagotomy, pancreatic insufficiency, sickle cell hemoglobinopathy, and hemolytic anaemia) or were receiving medications (e.g. morphine and morphine-related medications, atropine, calcium blockers, octreotide, progesterone, histamine 2 receptor stimulators, theophylline, glucagons and indomethacin) known to alter gallbladder contraction were excluded (Ziessman et al., 2001). The subjects were instructed not to take drugs affecting gallbladder (gastrointestinal) motility at least 10 days before the examination similar to a previous report in literature (Burkes, 2000). The fasting blood sugar estimation was conducted using a portable blood glucose meter (companion 2 metre; Medisense, Waltham, MA) on the first day. Subjects were advised not to drink water or any other thing after 6.30 am on the day of the examination and to report for each investigation after an overnight fast.

Erythromycin and azithromycin were supplied as tablets. Erythromycin (Medopharm, India) and azithromycin (SWISS Pharma PVT Ltd) were supplied in 500 mg. Immediately before the procedure, each subject took a tin of full cream peak brand milk (157 ml, 170 g, contents: vitamins and iodine, milk fat 9%, milk solids not fat 22%, milk stabilizer E339, brand of friesland foods, WAMCO Nig. Plc) followed by drinking of 30 cl of ion free water (Eva water, Coca Cola Co. Plc) (Arient et al., 1994). This amounted to 457 ml of liquid (milk and water) meal. One minute was allowed for both milk and water intake. Both milk and water were stored in a large flask at room temperature. The decision to adopt milk dilution was made by volunteers who indicated that they may have some sense of nausea if they take raw and undiluted milk.

Through a computer generated random numbers, the subjects were divided into two groups with 12 subjects in each group. Group A started with azithromycin and subsequently crosses over to group B, while group B started with erythromycin and subsequently after the "wash out" period crosses over to group A. At least a 10 day "wash out" period between the two interventions was adopted. The volunteers were scanned on two separate visits to compare the effects of erythromycin and azithromycin on gallbladder motility (contraction index). Subjects were examined between 0800 and 0900 after an overnight fast. Thirty minutes before the procedure (ingestion of test meal of milk and water), the subjects took the drug (500 mg erythromycin or 500 mg azithromycin) with 20 ml of water. This 30 min gap and 500 mg dosage have been used in a similar study (Acalovschi et al., 2002). Immediately before meal ingestion, gallbladder dimensions in three orthogonal planes were obtained in supine position to calculate the fasting gallbladder ellipsoid volume. GBCI was calculated using change in volume divided by original fasting volume multiplied by 100. Immediately after meal ingestion, the subject laid on the couch for postprandial measurements.

Established protocols were used to measure meal stimulated gallbladder emptying (Hussaini et al., 1996). The maximum length, width and height of the gallbladder were obtained in supine position as previously documented (Ugwu, 2006, 2008; Ugwu and Erundu,

Table 1. Comparative stimulation of gallbladder motility after administration of erythromycin and azithromycin.

Parameter	T ₅	T ₁₀	T ₁₅	T ₂₀	T ₂₅	T ₃₀	T ₃₅	T ₄₀
Mean GBCI (Erythromycin + fatty meal)	39.18	46.81	64.23	67.58	72.4	70.4	83.29	72.93
Mean GBCI (Azithromycin + fatty meal)	18.53	38.97	59.46	67.43	76.5	79.9	80.75	83.12
P	0.00	0.049	0.043	0.95	0.04	0.01	0.014	0.000

GBCI: Gallbladder contraction index in percentage; Tx: time at which GBCI was obtained in minutes.

2009). The gallbladder volume and emptying were assessed by the ellipsoid method (Dodds et al., 1986). Serial volume measurements were obtained immediately before the test meal, 5, 10, 15, 20, 25, 30, 35 and 40 min after the test meal. The 40 min adopted in this study have been used in a previous study (Elrichman et al., 2007). Subjects were allowed to sit down in between procedures but lay down (supine) during ultrasonography of the gallbladder. All observations were made by an imaging scientist experienced in gut sonography. The percentage changes in gallbladder sizes using the fasting volume as initial volume at every point were calculated as the gallbladder contraction indices (ejection fractions). The subjects ages were obtained, heights were measured on a calibrated vertical wall and weights obtained on a weighing scale (model H 89 LT Blue).

Statistical analyses were conducted using SPSS software version 16.0 (SPSS INC; Chicago, Illinois, USA). Gaussian responses of gallbladder contraction indices (GBCIs) were conducted using Kolmogorov-Smirnoff test. Paired (repeated measure) t-test were conducted to assess the differences in mean values of GBCIs at erythromycin and azithromycin phases. $P < 0.005$ was used as the criteria of statistical significance.

RESULTS

Twenty four males enrolled into and completed the study. Their ages ranged from 27 to 40 years, while their weight ranged from 55 to 69 kg.

Table 1 shows a comparative stimulation of gallbladder motility after administration of erythromycin and azithromycin.

In the first 15 min, erythromycin showed a significantly higher GBCI. This was not sustained after the 15th minute as the GBCI values in the 20th minute with both erythromycin and azithromycin were not significantly higher than GBCI values with erythromycin, but this was not sustained at the 35th minute as GBCI values were higher with erythromycin (Table 1). On the 40th minute, the GBCI values were higher with azithromycin.

DISCUSSION

Erythromycin is a prokinetic motilin analogue. Its treatment for animals on high cholesterol diet restored gallbladder contractility and reduced crystal formation (Xu et al., 1998). The result of this study indicated that erythromycin significantly enhanced gallbladder contractility compared to and better than azithromycin, having shown greater impact on GBCI at more stages (Table 1) than azithromycin. This enhancement of gallbladder motility by erythromycin has been reported previously (Xu et al.,

1998; Urbain et al., 1990). Hence, from efficacy point of view, erythromycin should be the preferred prokinetic macrolide to azithromycin, but from the tolerance point of view, azithromycin which showed cholestecytokinetic superiority in few stages could be used.

This novel finding indicates that azithromycin could be used as an alternative cholecystokinetic agent for the treatment of gallbladder dysmotility and perhaps cholestasis which can predispose to stone growth. This comparison was done for the purpose of assessing whether azithromycin can be used as a substitute to erythromycin in patients with known cardiac disease (Prolonged QT) or in patients on concomitant medications which also interact with the cytochrome P 450 enzymes like erythromycin does. Patients with unexplained abdominal pain are often on antidepressants, most of which interact with the CYP3A isoenzymes as erythromycin would induce them. In contrast, azithromycin does not inhibit the CYP3A isoenzymes.

Another benefit in using azithromycin is that it reaches higher intracellular concentrations, thus increasing both its duration of action and its efficacy (Moshiree et al., 2010). Furthermore, azithromycin is absorbed rapidly with food, increasing its absorption so that it can be taken with or without food. Moreover, in contrast to erythromycin, azithromycin has higher oral bioavailability. This bioavailability gives azithromycin an extensive distribution throughout the body with high tissue levels and at the same time poor central nervous system penetration.

Finally, the long half-life of azithromycin (up to 68 h) can potentially allow for easier administration due to long duration of effect (Moshiree, 2010). Azithromycin could be dosed once daily, as a result, as opposed to four times daily administration of oral erythromycin. This once daily may also improve patient compliance with taking the medication.

Future studies in this area involving chronic dosing of these drugs would further underpin their actual clinical value in cholecystokinesis and GBCI for $t > 40$ min should be ascertained using similar methodology with blinding of subjects and the sonographers conducting the study. On efficacy grounds, erythromycin is suggested as a preferred cholecystokinetic drug, but from tolerance point of view, azithromycin should be used.

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Full Length Research Paper

Protective effect of sildenafil against cysteamine induced duodenal ulcer in Wistar rats

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The aim of the current study was to investigate the possible protective effect of sildenafil (SIL) on cysteamine induced peptic ulcer in Wistar rats. Rats were randomly divided into five groups; six animals each. Normal control group; in which animals received an aqueous solution of Tween 80 (1 ml/kg) as a vehicle, two doses orally at an interval of 4 h. SIL group, in which animals received 25 mg/kg SIL orally 30 min before vehicle administration. Cysteamine group; in which duodenal ulceration was induced by two oral doses of cysteamine-HCl (450 mg/kg in 10% aqueous solution) at an interval of 4 h. Cysteamine+omeprazole group; in which animals were pretreated with 20 mg/kg omeprazole orally 30 min before cysteamine. Cysteamine+SIL group; in which animals were pretreated with 25 mg/kg SIL orally, 30 min before cysteamine. Twenty-four hours after the last dose of vehicle or cysteamine, rats were euthanized and the duodena were removed to determine the number of ulcers, ulcer surface area, ulcer score and ulcer index as well as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), myeloperoxidase (MPO), thiobarbituric acid reactive substances (TBARS) in tissue homogenates referring to the malondialdehyde, reduced glutathione (GSH) and oxidized glutathione (GSSG). The results of the present study showed that SIL treatment decreased the number of ulcers, the ulcer surface area, the ulcer score and the ulcer index in the cysteamine induced duodenal ulcer. Moreover, SIL ameliorated the biochemical changes that were induced by cysteamine. In conclusion, SIL attenuates experimentally induced peptic ulceration using cysteamine partially through induction of nitrogen oxide (NO) and antioxidant effect which may be useful in the treatment of cystinosis.

Key words: Cysteamine, cystinosis, sildenafil, duodenal ulcer, rats.

INTRODUCTION

Peptic ulcer disease is an ulcerative gastrointestinal disease affecting the stomach and duodenum and causes a high rate of morbidity (Blanton et al., 2001). Duodenal ulcer is the most common type of peptic ulcer where discontinuity in the gastric mucosa is commonly observed. It is clear that gastric acid and pepsin secretion are

necessary in its pathogenesis, however, factors related to mucosal resistance particularly the production of gastrointestinal mucus and secretions of bicarbonate are also important (Katzung, 2001).

Cysteamine hydrochloride has been found to be the most potent agent for inducing duodenal ulcer, and

cysteamine induced duodenal ulcer in animals is now used to study the antiulcer activity of drugs (Szabo, 1978; Minaiyan et al., 2005). The cysteamine used in experimental studies has been found to concentrate in the duodenum (Nakamura et al., 2008). Its ulcerogenic effect may be due to the generation of reactive oxygen species (ROS), the decreasing defense activity of superoxide dismutase (SOD) and increasing duodenal endothelin-1 concentration, which are all associated with decreased duodenal mucosal blood flow (Jeitner and Lawrence, 2001; Khornenko et al., 2003). Oxidative stress, enhanced free-radical levels, and an impaired in-cell antioxidant pool are important factors underlying the pathophysiologic mechanisms in a variety of diseases (Khornenko et al., 2003).

In addition, intragastric nitric oxide (NO) has been shown to stimulate gastric blood flow and mucus generation (Bjorne et al., 2004) and to modulate secretory functions (Holm et al., 2000). NO and cyclic guanosine monophosphate (cGMP) were shown to protect parietal cells from ethanol-induced cytotoxicity (Yanaka et al., 1995). Moreover, NO/cGMP pathway was proven to protect endothelial cells against cellular damage in various tissues (Polte et al., 1997).

Sildenafil (SIL) increases the effects of cGMP by blocking the phosphodiesterase-type 5 (PDE-5), which inactivates intracellular cGMP, the second messenger in the NO signaling pathway (Chuang et al., 1998). Recently, several studies have shown that, in addition to treating erectile dysfunction, sildenafil can prevent or decrease tissue injury. Two previous studies demonstrated the beneficial anti-inflammatory effect of SIL on acetic acid-induced acute colitis and bleomycin-induced lung fibrosis models in rats via prevention of lipid peroxidation, oxidant generation, cytokine production and neutrophil accumulation (Iseri et al., 2009; Yildirim et al., 2010).

Sildenafil has been shown previously to protect against both indomethacin-induced gastropathy (Santos et al., 2005) and ethanol-induced gastric damage (Medeiros et al., 2008). However, its protective effect against cysteamine-induced duodenal ulcer has not been investigated. So, the aim of the current study is to investigate the possible protective effect of SIL on cysteamine induced peptic ulcer in Wistar rats.

MATERIALS AND METHODS

Drugs and chemicals

Sildenafil (Viagra; Pfizer, NY, USA) tablets (50 mg) were obtained from commercially available sources and dissolved in tap water. Drug solution was administered to the animals in a volume of 0.5 ml by means of an orogastric tube. Omeprazole (Losec, 20 mg tablets) was a kind gift from AstraZeneca, Riyadh, Saudi Arabia. Superoxide dismutase (SOD) kit (Ransod) and glutathione peroxidase (GSH-PX) kit (Ransel) were purchased from (Randox

Laboratories Ltd., Crumlin Co. Antrim, UK). Cysteamine-HCl and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male adult Wistar rats, weighing 200 to 250 g were obtained from the animal facility of King Fahd Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. They were used in the study according to the guidelines of the Biochemical and Research Ethics Committee at King Abdulaziz University, in accordance with the National Institutes of Health (NIH) guidelines. Animals were kept on wire mesh floors to prevent coprophagy and were housed in a well-ventilated, temperature-controlled room at $22 \pm 2^\circ\text{C}$ with a 12 h light-dark cycle. The food consisted of the normal rat chow and water was provided *ad libitum*. Care was taken to avoid stressful conditions. All experimental procedures were performed between 8 and 12 a.m to avoid diurnal variations of putative regulators of gastric functions.

Experimental design

Duodenal induced peptic ulcer

Rats were fasted for 24 h prior to the experiment and randomly divided into five groups; six animals each:

1. Normal control group; in which animals received an aqueous solution of Tween 80 (1 ml/kg) as a vehicle, two doses orally at an interval of 4 h.
2. SIL group; in which animals received 25 mg/kg SIL orally, 30 min before vehicle administration.
3. Cysteamine group; in which duodenal ulceration was induced by two oral doses of cysteamine-HCl (450 mg/kg in 10% aqueous solution) at an interval of 4 h according to the previously described method by Szabo et al. (1979).
4. Cysteamine+omeprazole group; in which animals were pretreated with 20 mg/kg omeprazole orally, 30 min before cysteamine (Desai et al., 1997).
5. Cysteamine+SIL group; in which animals were pretreated with 25 mg/kg SIL orally, 30 min before cysteamine. The dose of SIL was chosen according to the previous study by Karakoyun et al. (2011) which revealed efficacy as anti-inflammatory and antiapoptotic in an experimentally induced colitis.

Twenty-four hours after the last dose of vehicle or cysteamine, rats were euthanized with an ether overdose and the duodena (5 cm in length) were removed carefully. The duodenum was incised along the antimesenteric side and rinsed with saline; ulcers were examined under 5-fold binocular magnification to assess lesions. The number of ulcers, ulcer surface area, ulcer score and ulcer index were determined. Ulcer intensities were scored using a 4-point scale (0 = no lesion, 1 = superficial mucosal erosion, 2 = deep ulcer or transmural ulcer, 3 = perforated or penetrated ulcer) (Szabo et al., 1979; Vogel, 1997). The ulcer index was calculated by the following equation:

$$U_i = U_N + U_S + U_A \times 0.1$$

Where U_i is the ulcer index, U_N is the ulcer number, U_S is the ulcer score and U_A is the ulcer surface area for each duodenum. The ulcer surface area was estimated using transport tapes fixed on

Table 1. The effect of sildenafil (SIL) on cysteamine induced alterations in ulcer score, ulcer surface area and ulcer index.

Parameter	Ulcer score	Ulcer surface area (mm ²)	Ulcer index
Normal group	0	0	0
SIL group	0	0	0
Cysteamine group	2.32±0.20*	58.24±4.21*	12.30±1.10*
Omeprazole + cysteamine group	0.70±0.13*#	16.14±1.50*#	3.42±0.46*#
SIL + cysteamine treated group	0.80±0.11*#	20.12±2.02*#	5.46±0.62*#

Data are the mean ± SD of 6 rats. **p* < 0.05 versus corresponding control group. #*p* < 0.05 versus corresponding cysteamine group.

a light, transparent sheet. The tape transport was divided into cells each 1 mm² in area; the number of cells was counted and the ulcer area was thus measured for each duodenum (Szabo et al., 1979).

Biochemical assays in duodenal tissues

Duodenal tissue samples were weighed and 0.5 g samples were homogenized and the homogenates were centrifuged for 15 min at 17,000 rpm.

The supernatants were collected and kept frozen at -80°C for subsequent biochemical studies. SOD activity in duodenal tissue was assayed as described in the Randox-Ransod enzyme kit. SOD activity was determined according to the method of Sun et al. (1988). The principle of the method is based on the inhibition of nitroblue-tetrazolium reduction by the xanthine-xanthine oxidase system as a superoxide generator. Values are expressed as U/mg protein.

Glutathione peroxidase (GSH-PX) activity was assessed spectrophotometrically as described in the Randox-Ransod enzyme kit. GSH-Px activity was assessed spectrophotometrically according to the method of Paglia and Valentine (1967) and expressed as U/mg protein. Myeloperoxidase (MPO) activity was determined by the method of Wei and Frenkel (1993). The principle of the assay is based on using 4-aminoantipyrene/phenol solution as the substrate for MPO-mediated oxidation by H₂O₂ and recording the changes in absorbance at 510 nm. Values are expressed as mU/g protein. Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) in tissue homogenates referring to the malondialdehyde (MDA) standard calibration curve according to the method of Mihara and Uchiyama (1978). Values are expressed as nmol/g protein. Reduced glutathione (GSH) content and oxidized glutathione (GSSG) level were assessed according to the modified method of Ellman (1959) and values are expressed as nmol/mg protein.

Statistical analysis

The data were analyzed statistically by t-test and expressed as mean ± standard deviations (SD). Student t-test was used to check the statistical significance of the observed differences in mean values. All calculations were performed using Software GraphPad InStat, Version 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was determined as *p* value below 0.05.

RESULTS

No ulcer or erosions were observed in normal or SIL rats

except for mild hyperemia in the SIL group indicating that SIL had no interference with experimental outputs. Cysteamine has induced peptic ulceration in rats that was revealed by a significant increase in ulcer score, ulcer surface area and ulcer index. These changes were significantly reduced in ulcer induced rats pretreated with either omeprazole or SIL (Table 1). Moreover, cysteamine significantly decreased both SOD and GSH-Px activity and increased MPO activity. These changes were significantly ameliorated in ulcer induced rats pretreated with either omeprazole or SIL (Table 2). In addition, cysteamine significantly increased both TBARS and GSSG compared with the control group and decreased GSH levels. These changes were ameliorated to near normal levels in ulcer induced rats pretreated with either omeprazole or SIL (Table 2 and Figure 1).

DISCUSSION

The only existing treatment for cystinosis is the aminothiols cysteamine, a drug that reduces intracellular cystine levels (Pisoni et al., 1995). Oral cysteamine therapy, if administered early in the course of the disease, delays the progression of renal insufficiency (Markello et al., 1993), however, it may cause digestive intolerance especially duodenal ulcer (Gahl et al., 1987). On the other hand, SIL has shown previously to protect against gastric damage induced by either indomethacin (Santos et al., 2005) or ethanol (Medeiros et al., 2008).

In the present study, cysteamine was used as a cytotoxic agent to induce duodenal ulcer which was evident by an increase in the ulcer score, ulcer surface area and ulcer index. The cysteamine model of duodenal ulceration in rats was first described by Selye and Szabo (1973). The mechanism of pathogenesis in the cysteamine-induced duodenal ulcer model may be due to a hypersecretion of gastric acid, deterioration of mucosal resistance and promotion of gastric emptying (Szabo et al., 1979; Lichtenberger et al., 1977; Briden et al., 1985). Moreover, the mechanism may involve the generation of hydroxyl radical, and it has been suggested that

Table 2. The effect of sildenafil (SIL) on cysteamine induced alterations in duodenal superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and myeloperoxidase (MPO).

Parameter	SOD (U/mg protein)	GSH-PX (U/mg protein)	MPO (mU/g protein)
Normal group	5.2±0.42	8.4±0.80	6.6±0.46
SIL group	5.8±0.48	8.6±0.78	6.8±0.42
Cysteamine group	0.9±0.08*	4.8±0.42*	10.2±0.81*
Omeprazole + cysteamine group	1.8±0.02 [#]	5.2±0.52	8.8±0.83
SIL + cysteamine treated group	1.2±0.01 [#]	7.9±0.65 [#]	7.2 ± 0.63 [#]

Data are the mean ± SD of 6 rats.**p* < 0.05 versus corresponding control group; [#]*p* < 0.05 versus corresponding cysteamine group.

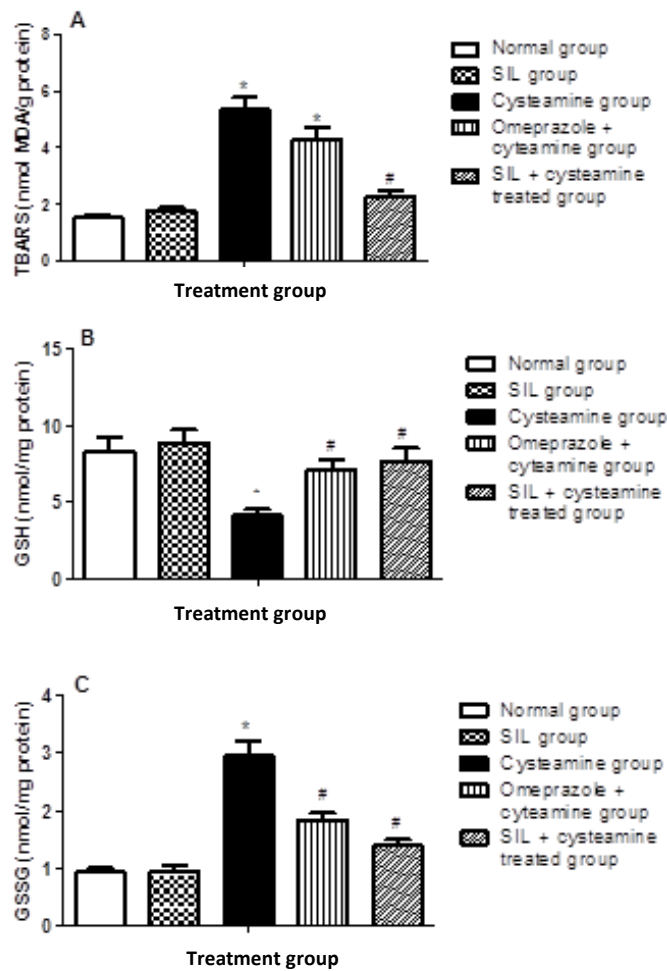


Figure 1. The effect of sildenafil (SIL) on cysteamine induced alterations in thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), and oxidized glutathione (GSSG). Data are the mean ± SD of 6 rats.**p* < 0.05 versus corresponding control group [#]*p* < 0.05 versus corresponding cysteamine group.

antioxidants can inhibit cysteamine-induced duodenal ulcer (Khomenko et al., 2003). Its ulcerogenic effect may

be due to the generation of ROS decreasing defense activity of SOD (Jeitner and Lawrence, 2001; Khomenko

et al., 2003). This is in accordance with the present study which revealed a decrease in the SOD activity in the cysteamine treated group in addition to decrease in the GSH-PX and GSH, with an increase in the GSSG.

In the present study, cysteamine caused an elevation in duodenal MPO activity, indicating the presence of enhanced polymorph nuclear leukocyte recruitment in the inflamed tissue, while the increased duodenal TBARS level, an indicator of lipid peroxidation, verified the oxidative damage in the renal tissue (Klebanoff, 2005).

In the current study, pretreatment with SIL ameliorated the increase in the MPO activity which is consistent with Santos et al. (2005) who showed that SIL provides effective protection against indomethacin-induced gastropathy in rats via preventing the effect of indomethacin on gastric blood flow and MPO activity. Moreover, pretreatment with SIL reduced the ulcer indices and TBARS level and elevated the activities of SOD and GSH-PX. These results are consistent with those of Karakoyun et al. (2011) who concluded that SIL is beneficial in experimentally-induced rat colitis partially by nitric oxide-dependent mechanisms via the maintenance of oxidant-antioxidant status.

Sildenafil is a selective inhibitor of phosphodiesterase-5 (PDE5), which degrades cyclic guanosine monophosphate (cGMP), a downstream product in the NO-soluble guanylate cyclase cascade, in endothelial cells. Interestingly, sildenafil can induce iNOS and eNOS, and sildenafil-induced increases in NO have been associated with attenuation of doxorubicin-induced cardiotoxicity (Ockaili et al., 2002). NO plays an important role in the host defense and inflammation response. It also modulates several elements of gastric mucosal defense, including blood flow (Whittle et al., 1981), neutrophil adhesion (Kubes et al., 1991; May et al., 1991) and mucus secretion (Allen et al., 1993; Wallace and Miller, 2000). The effect of NO is at least partially mediated by an increase in cGMP content, and cGMP is normally broken down rapidly by PDE-5. In addition, NO acts as an endogenous mediator of the gastroprotective effect of different anti-ulcer agents (Brzozowski et al., 1999).

Cystinosis is usually associated with renal tubular atrophy (Gubler et al., 1999). Cysteamine which is used in the treatment of cystinosis enters the lysosome by a specific transporter for aminothiols or aminosulfides (Pisoni et al., 1995). Lee et al (2009) suggested that SIL has a renoprotective effect and attenuates experimental cisplatin-induced nephrotoxicity by preventing apoptosis. This finding with the findings of the current study may endorse further clinical investigations in human to evaluate the possible usefulness of SIL with cyteamine in cases of cystinosis to prevent its possible ulcerogenic effect on the duodenum and prevent the detrimental effect of cystinosis on the kidney.

Conclusion

SIL attenuates experimentally induced peptic ulceration using cysteamine partially through induction of NO and antioxidant effect. Further investigations may be needed to evaluate the possible clinical effect of SIL in patients with cystinosis and its clinical protective effect against duodenal ulcer.

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Full Length Research Paper

Antioxidative properties and inhibition of key enzymes linked to type-2 diabetes by snake tomato (*Tricosanthes cucumerina*) and two tomato (*Lycopersicon esculentum*) varieties

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This study sought to compare the antioxidant properties [1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl (OH) radicals scavenging abilities] and inhibition of Fe²⁺-induced lipid peroxidation and two key enzymes relevant to type-2 diabetes (α -amylase and α -glucosidase) of snake tomato (*Trichosanthes cucumerina*) with two tomato varieties [*Lycopersicon esculentum* Mill. var. *esculentum* (ESC) and *Lycopersicon esculentum* Mill. var. *cerasiforme* (CER)]. Snake tomato (0.84 mg/g) and CER (0.87 mg/g) had significantly ($P < 0.05$) higher total phenolic content than ESC (0.27 mg/g). However, CER had the highest total flavonoid content of 0.48 mg/g, compared to snake tomato (0.27 mg/g) and ESC (0.15 mg/g). In consonance with the phenolic content, CER and snake tomato had higher DPPH and OH radicals scavenging abilities than ESC. The inhibition of Fe²⁺ induced malondialdehyde (MDA) production in rats pancreas revealed that snake tomato had significantly lower inhibitory effect than CER. Furthermore, snake tomato and CER showed stronger inhibition of α -glucosidase [snake tomato ($EC_{50} = 1.65$ mg/ml), CER ($EC_{50} = 1.32$ mg/ml)] than α -amylase [snake tomato ($EC_{50} = 2.15$ mg/ml), CER ($EC_{50} = 2.39$ mg/ml)] activity. The antioxidant properties of snake tomato favourably compared with the cultivars of tomatoes, and its stronger inhibition of α -glucosidase activity than α -amylase activities suggests that snake tomato could be an alternative or complement to the use of lycopersicon tomatoes.

Key words: Tomato, antioxidant, diabetes, flavonoids, α -amylase, α -glucosidase.

INTRODUCTION

Tricosanthes cucumerina (commonly called snake tomato), snake gourd, viper gourd or long tomato is rich in chemical constituents like flavonoids, carotenoids, phenolic acids (Adebooye, 2008; Ojiako and Igwe, 2008)

which makes the plant pharmacologically and therapeutically active. Even though some systems of medicine have been exploring some pharmacological potentials of the snake tomato, such as antidiabetic,

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hepatoprotective, cytotoxic, anti-inflammatory and larvicidal effects, the 'tomato' still remains underutilized as food or as medicinal plant. Snake tomato has been used as a substitute for the common lycopersicon tomatoes in the tropics especially when the prices of the lycopersicon tomatoes go up in the off-season. The common tomatoes commonly used as diet almost all over the world, are a major source of antioxidants, and the consumption of fresh lycopersicon tomatoes has been reported to have health benefits such as cancer prevention and inhibition of lipid peroxidation (Bub et al., 2000; Ziegler and Vogt, 2002). However, there is dearth of information on some health benefits of snake tomato to justify its use as a substitute to the lycopersicon tomatoes.

The link between free-radicals and development of diabetes has been well established (Ceriello, 2006; Maritim et al., 2003), more so free-radical damage to the pancreas has been implicated in the diabetogenic process (Akbarzadeh et al., 2007). Diabetes is a major health problem worldwide along with its associated complications (Zimmet et al., 1997) and this could be linked to changes in the dietary patterns in both developing and developed countries. The prevalence of type II diabetes is growing at an exponential rate (Zimmet and Lefebvre, 1996) and a lot of attention is been given to natural products for the management of the disease (Covington, 2001).

This study therefore investigated the antioxidant properties and inhibition of key enzymes linked to type-2 diabetes (α -amylase and α -glucosidase) by the underutilized snake tomato and compare with the common tomatoes so as to find a basis, if any, for the use of snake tomato as a substitute to common tomatoes and also explain the mechanism of action by which the 'tomatoes' can be used in the management of type-2 diabetes.

MATERIALS AND METHODS

Sample collection and preparation

Snake tomato (*T. cucumerina*) and two cultivars of common tomatoes (*Lycopersicon esculentum* Mill.); Ibadan Local (CER) and Roma VF (ESC) were collected from the main market, identified, washed, weighed and then homogenized by using a blender after distilled water was added (1:3 w/v). The homogenate was centrifuged at 4500 g for 15 min. The supernatant (juice fraction) was recovered and kept in the freezer for subsequent analysis.

Determination of total phenol content

The total phenol content was determined according to the method of Singleton et al. (1999). Briefly, appropriate dilutions of the pastes were oxidized with 2.5 ml 10% Folin-Ciocalteu's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured at 765 nm in the spectrophotometer. The total phenol content was subsequently calculated as gallic acid equivalent.

Determination of total flavonoid content

The total flavonoid content was determined using a slightly modified method reported by Meda et al. (2005), briefly 0.5 ml of appropriately diluted sample was mixed with 0.5 ml methanol, 50 μ l 10% $AlCl_3$, 50 μ l 1 M potassium acetate and 1.4 ml water, and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm; the total flavonoid content was subsequently calculated. The non-flavonoid polyphenols were taken as the difference between the total phenol and total flavonoid content.

1,1-diphenyl-2-picrylhydrazyl radical scavenging ability

The free radical scavenging ability of the pastes against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was evaluated as described by Gyamfi et al. (1999). Briefly, appropriate dilution of the extracts (1 ml) was mixed with 1 ml, 0.4 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free radical scavenging ability was subsequently calculated.

Hydroxyl radical scavenging ability

The method of Halliwell and Gutteridge (1981) was used to determine the ability of the pastes to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose. The extract 0 to 100 μ l was added to a reaction mixture containing 120 μ l of 20 mM deoxyribose, 400 μ l of 0.1 M phosphate buffer, 40 μ l of 500 μ M of $FeSO_4$, and the volume were made up to 800 μ l with distilled water. The reaction mixture was incubated at 37°C for 30 min and the reaction was then stopped by the addition of 0.5 ml of 28% trichloroacetic acid. This was followed by addition of 0.4 ml of 0.6% thiobarbituric acid solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm in a spectrophotometer.

Lipid peroxidation assay

Preparation of tissue homogenates

Adult male rats weighing 220 to 240 g (10 to 12 weeks old) were decapitated under mild diethyl ether anaesthesia and the pancreas was rapidly isolated and placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1/10 w/v) with about 10-up-and-down strokes at approximately 1200 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3000 \times g to yield a pellet that was discarded, and a low-speed supernatant (S1) was kept for lipid peroxidation assay (Belle et al., 2004).

Lipid peroxidation and thiobarbituric acid reactions

The lipid peroxidation assay was carried out using the modified method of Ohkawa et al. (1979), briefly 100 μ l S1 fraction was mixed with a reaction mixture containing 30 μ l of 0.1 M pH 7.4 Tris-HCl buffer, extract (0 to 100 μ l) and 30 μ l of 250 μ M freshly prepared $FeSO_4$. The volume was made up to 300 μ l by water before incubation at 37°C for 1 h. The colour reaction was developed by adding 300 μ l 8.1% Sodium dodecyl sulphate (SDS)

to the reaction mixture containing S1, this was subsequently followed by the addition of 600 μ l of acetic acid/HCl (pH 3.4) mixture and 600 μ l 0.8% Thiobarbituric acid (TBA). This mixture was incubated at 100°C for 1 h. Thiobarbituric acid reactive species (TBARS) produced were measured at 532 nm and the absorbance was compared with that of standard curve using malondialdehyde (MDA).

α -Amylase inhibition assay

This was measured using the dinitrosalicylic acid method adapted from Bernfeld (1955). Appropriate dilution of the pastes (500 μ l) and 500 μ l of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing Hog pancreatic α -amylase (EC 3.2.1.1) (0.5 mg/ml) were incubated at 25°C for 10 min. Then, 500 μ l of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixtures was incubated at 25°C for 10 min and stopped with 1.0 ml of dinitrosalicylic acid colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 10 ml of distilled water, and absorbance measured at 540 nm. The EC₅₀ (the extract concentration inhibiting 50% of the α -amylase activity) of the pastes was calculated.

α -Glucosidase inhibition assay

Appropriate dilution of the pastes (50 μ l) and 100 μ l of α -glucosidase solution (1.0 U/ml) in 0.1 M phosphate buffer (pH 6.9) was incubated at 25°C for 10 min. Then, 50 μ l of 5 mM p-nitrophenyl- α -D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added. The mixtures were incubated at 25°C for 5 min before reading the absorbance at 405 nm in the spectrophotometer. The α -glucosidase inhibitory activity was expressed as percentage inhibition. The EC₅₀ of the pastes was calculated (Apostolidis et al., 2007).

Data analysis

The results of three replicates were pooled and expressed as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) and least significance difference (LSD) were carried out (Zar, 1984). Significance was accepted at $p \leq 0.05$.

RESULTS

The results of the total phenolic and flavonoid contents are presented in Table 1. ESC had significantly lower total phenolic content than snake tomato and CER, while CER had significantly higher total flavonoid content than snake tomato and ESC. Figures 1, 2 and Table 2 reveal that CER and snake tomato had higher radicals scavenging abilities than ESC. The inhibition of Fe²⁺ induced MDA production in rats pancreas is presented in Figure 3 and Table 2. Snake tomato had significantly lower inhibitory effect than CER, but higher inhibitory effect than ESC. Furthermore, as presented in Figures 4, 5 and Table 2, snake tomato and CER showed stronger inhibi-

Table 1. Total phenol and total flavonoid content of snake tomato and two tomato varieties (ESC and CER) (mg/g).

Sample	Total phenol	Total flavonoid
Snake tomato	0.84 \pm 0.11 ^a	0.27 \pm 0.05 ^a
ESC	0.27 \pm 0.06 ^b	0.15 \pm 0.02 ^b
CER	0.87 \pm 0.08 ^a	0.48 \pm 0.04 ^c

Values represent means of triplicate (n=3). Values with the same alphabet (a,b,c) along the same column are not significantly different ($P > 0.05$). ESC - *Lycopersicon esculentum* Mill. var. *esculentum*. CER - *Lycopersicon esculentum* Mill. var. *cerasiforme*

tion of α -glucosidase than α -amylase activity.

DISCUSSION

The total phenol content of snake tomato was not significantly ($P > 0.05$) different from that of CER, but higher than that of ESC. However, the total flavonoid content of snake tomato was significantly ($P < 0.05$) lower than that of CER, but higher than that of ESC. The antioxidant capacities of the 'tomato' samples were assessed not only for comparison between the species but also because free radicals are involved in the development and complications of diabetes in a number of ways; the white blood cell production of reactive oxygen species mediates the autoimmune destruction of the beta cells in the islets of langerhans in the pancreas (Yoon and Jun, 2005). Abnormalities in transition metal metabolism are postulated to result in the establishment of diabetes (Parameshwar et al., 2012). Diabetes can be induced in animals by the drugs alloxan and streptozotocin; the mechanism of action of these two drugs is different, but both result in the production of reactive oxygen species and scavengers of oxygen radicals have been found to be effective in preventing diabetes in these animal models (Moussa, 2008).

The DPPH* scavenging ability of snake tomato was not significantly ($P > 0.05$) different from ESC, but lower than that of CER. This trend in the results agree with the total phenolic and flavonoid content of the samples and many earlier research articles, where correlation were reported between phenolic content and antioxidant capacity of some plant foods (Amic et al., 2003; Chu et al., 2002; Oboh and Ademosun, 2011). Furthermore, the trend in the DPPH* scavenging ability agrees with the OH* scavenging ability of the samples. All the samples scavenged OH* produced from the decomposition of deoxyribose in a dose dependent manner but with CER and snake tomato having the highest scavenging abilities as there was no significant ($P > 0.05$) difference in their EC₅₀. The antioxidant properties of the samples could be

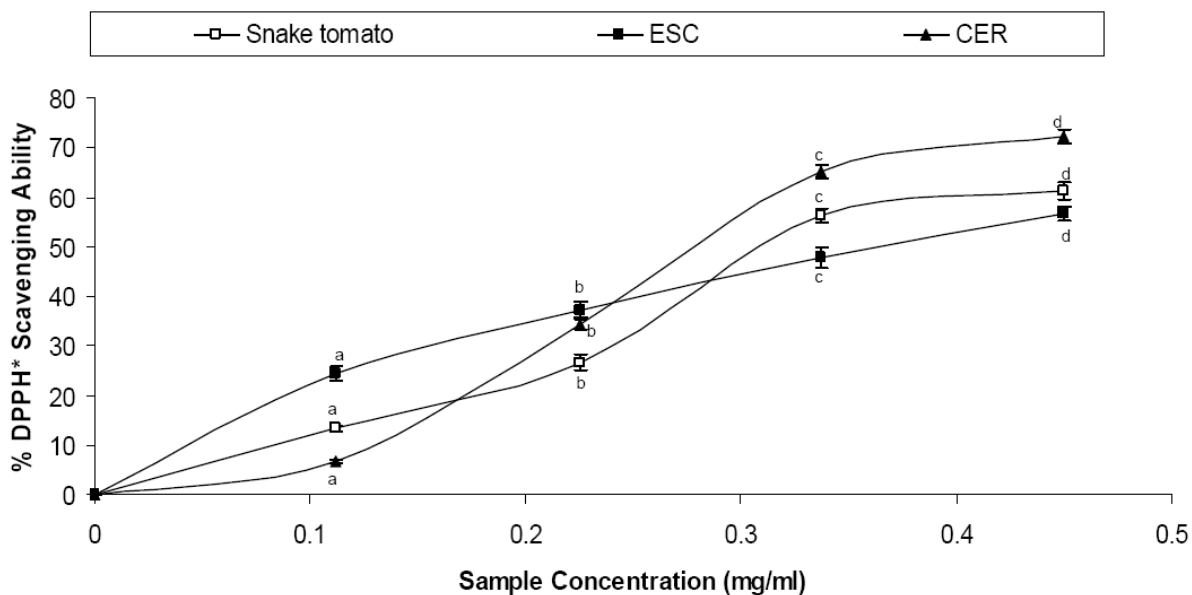


Figure 1. DPPH* scavenging abilities of snake tomato and two tomato varieties (ESC and CER). ESC - *Lycopersicon esculentum* Mill. var. *esculentum*. CER - *Lycopersicon esculentum* Mill. var. *cerasiforme*.

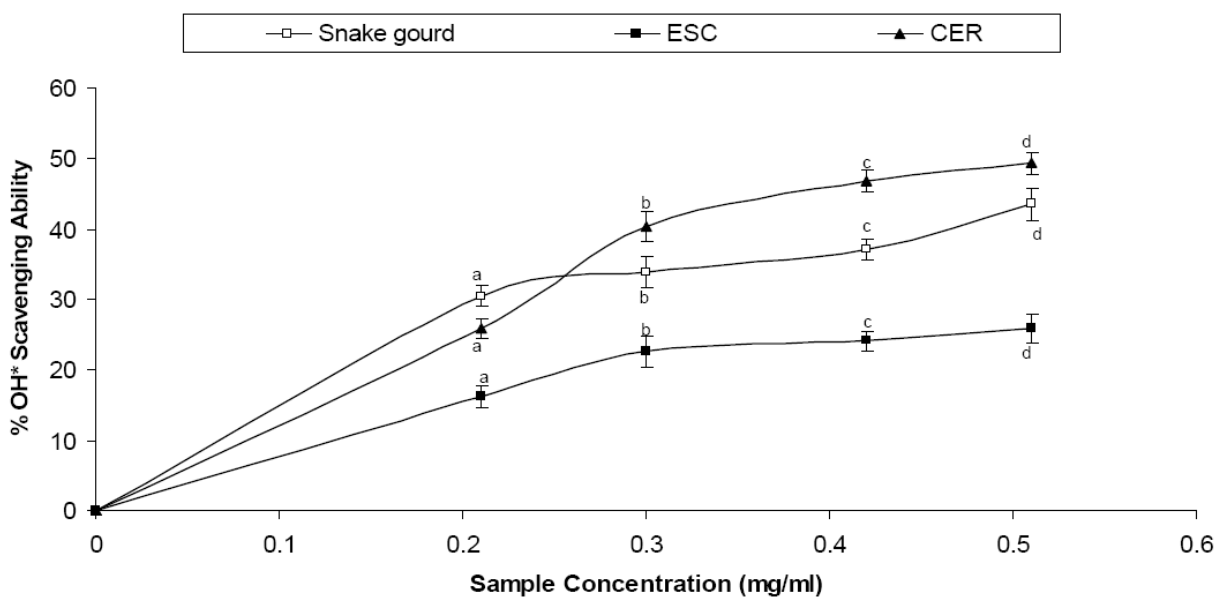


Figure 2. OH* scavenging abilities of snake tomato and two tomato varieties (ESC and CER). ESC - *Lycopersicon esculentum* Mill. var. *esculentum*. CER - *Lycopersicon esculentum* Mill. var. *cerasiforme*

attributed to a combination of carotenoids and other phenolic compounds. Lycopene, which is the major carotenoid in tomatoes has been shown by cellular and molecular studies to exhibit potent antioxidative properties (Khachik et al., 2002; Sahasrabudde, 2011), and correlations have been established between the

phenolic content and antioxidant properties of many samples (Jayaprakasha et al., 2008; Kedage et al., 2007).

MDA is increased when Fe^{2+} induces lipid peroxidation by catalyzing the decomposition of hydrogen peroxide to generate hydroxyl radical via the fenton reaction (Bayir et

Table 2. EC₅₀ of DPPH* and OH* scavenging abilities, inhibition of Fe²⁺ induced MDA Production, α-amylase and α-glucosidase activities of snake tomato and two tomato varieties (ESC and CER).

Sample	DPPH* (mg/ml)	OH* (mg/ml)	MDA (mg/ml)	α-amylase (mg/ml)	α-glucosidase (mg/ml)
Snake tomato	0.35±0.09 ^a	0.52±0.07 ^a	5.51±0.09 ^a	2.15±0.09 ^a	1.65±0.14 ^a
ESC	0.36±0.08 ^a	0.84±0.08 ^b	6.71±0.06 ^b	1.81±0.06 ^b	1.93±0.11 ^b
CER	0.30±0.06 ^b	0.46±0.05 ^a	4.31±0.07 ^c	2.39±0.07 ^c	1.32±0.10 ^c

Values represent means of triplicate (n=3). Values with the same alphabet (a,b,c) along the same column are not significantly different (P>0.05). OH* - hydroxyl radical, MDA - malondialdehyde, DPPH* - 1,1-diphenyl-2-picrylhydrazyl radical, ESC - *Lycopersicon esculentum* Mill. var. *esculentum*, CER - *Lycopersicon esculentum* Mill. var. *cerasiforme*.

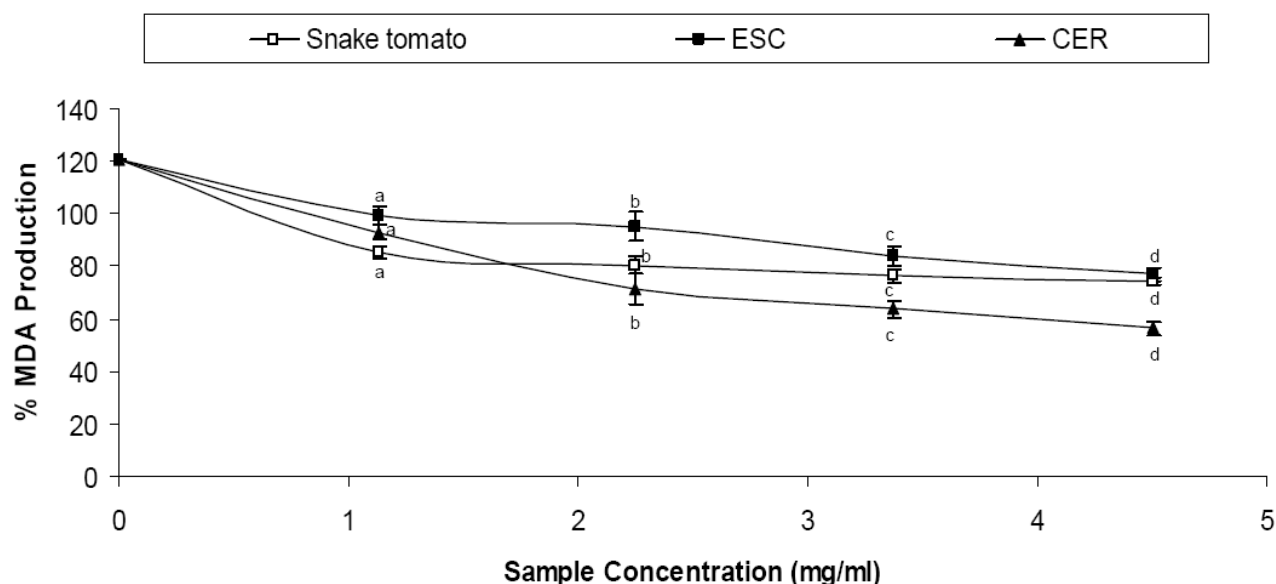


Figure 3. Inhibition of Fe²⁺ induced MDA production by snake tomato and two tomato varieties (ESC and CER). ESC - *Lycopersicon esculentum* Mill. var. *esculentum*. CER - *Lycopersicon esculentum* Mill. var. *cerasiforme*.

al., 2006; Oboh et al., 2007). Incubation of rat's pancreas in the presence of 250 μM Fe²⁺ caused a significant increase in the malondialdehyde (MDA) content of the pancreas. Free-radicals induced pancreatic damage has been linked to the development of diabetes (Akbarzadeh et al., 2007). Nevertheless, the 'tomato' samples significantly (P<0.05) inhibited MDA production in the pancreas in a dose-dependent manner as shown in Table 2. However, CER and snake tomato had significantly (P < 0.05) stronger inhibitory effect on MDA production in the pancreas (*in vitro*) than ESC. The reason for the higher inhibitory ability of CER and snake tomato cannot be categorically stated, but it could be due to other antioxidant mechanisms, since CER and snake tomato had higher phenolic contents, DPPH* and OH* scavenging abilities.

The MDA inhibitory ability of snake tomato and the lycopersicon tomatoes cannot be ascribed to the lycopene content alone as a combination of purified lycopene

with α-tocopherol was reported to result in a significant greater inhibition of *in vitro* low density lipoprotein (LDL) oxidation, than the expected additive individual inhibitions. Purified lycopene was also shown to act synergistically with other natural antioxidants like the flavonoid glabridin, the phenolics rosmarinic acid and carnosic acid, and garlic in inhibiting LDL oxidation *in vitro* (Furhman et al., 2000). Thus, the combination of lycopene and other natural antioxidants present in the samples may be responsible for the potent inhibition of lipid peroxidation.

α-Amylase hydrolyzes starch to maltose, while α-glucosidase enzymes are responsible for the breakdown of oligo- and/or disaccharides to monosaccharides, and an inhibition of these enzymes therefore leads to a decrease in blood glucose level, and this is one of the therapeutic approaches for reducing postprandial blood

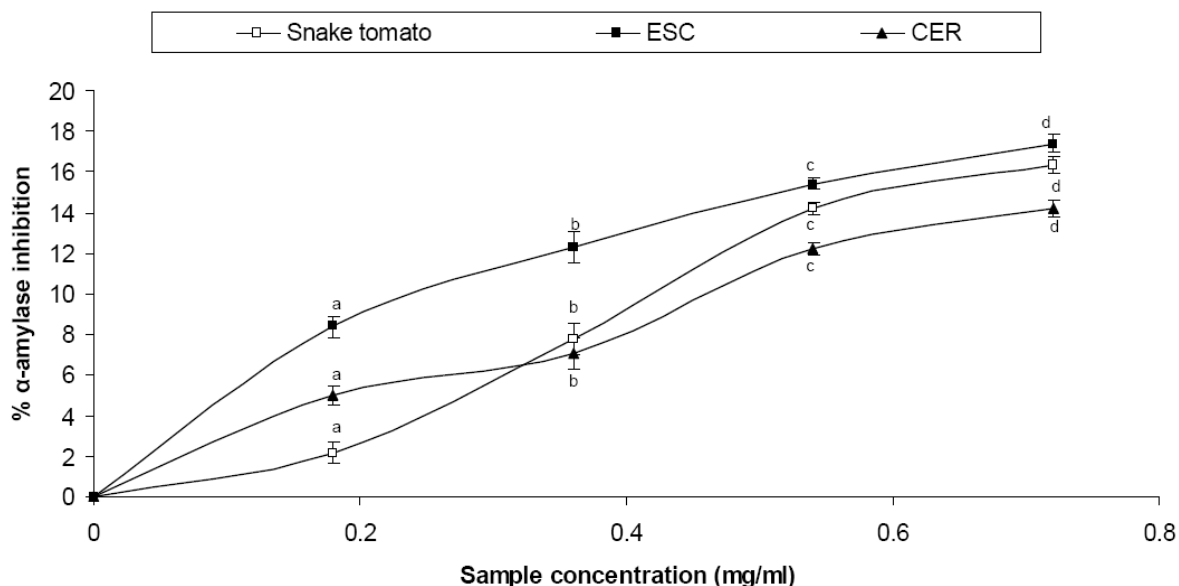


Figure 4. α -Amylase inhibitory ability of snake tomato and two tomato varieties (ESC and CER). ESC - *Lycopersicon esculentum* Mill. var. *esculentum*. CER - *Lycopersicon esculentum* Mill. var. *cerasiforme*.

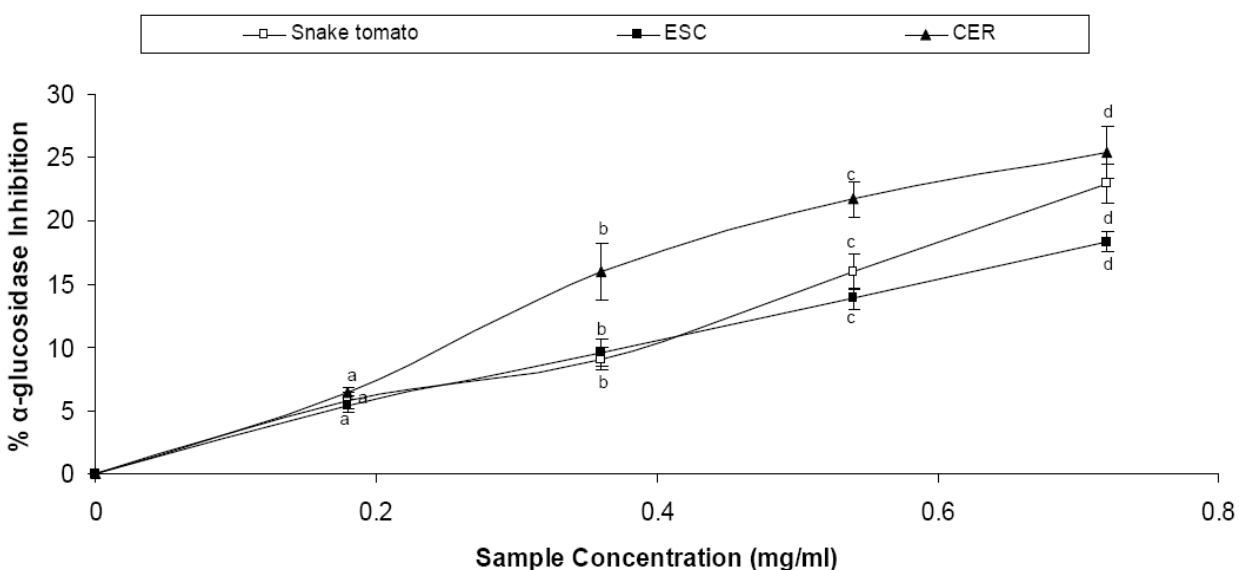


Figure 5. α -Glucosidase inhibitory ability of snake tomato and two tomato varieties (ESC and CER). ESC - *Lycopersicon esculentum* Mill. var. *esculentum*. CER - *Lycopersicon esculentum* Mill. var. *cerasiforme*.

glucose values in a bid to prevent/manage diabetes (Shim et al., 2003). The samples inhibited α -amylase activity in a dose-dependent manner, however the EC_{50} (Table 2) revealed that the trend of the result was different from the earlier results as ESC had the highest inhibitory effect. It is noteworthy however that snake

tomato had higher inhibitory effect on α -amylase activity than CER. Furthermore, the α -glucosidase inhibitory activity showed that the samples inhibited the enzyme activity in a dose-dependent manner.

A comparison of the α -amylase and α -glucosidase inhibition by the samples revealed a stronger inhibition of

α -glucosidase activity, but milder inhibition of α -amylase activities by snake tomato and CER. A stronger inhibition of α -glucosidase activity, but milder inhibition of α -amylase activities is desirable as this could address the main drawback of currently used α -glucosidase and α -amylase inhibitors drugs which is caused by the excessive inhibition of pancreatic α -amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Rao and Jamil, 2011). These results confirms the claim that natural inhibitors from dietary plants could show lower inhibitory effect against α -amylase activity and a stronger inhibitory activity against α -glucosidase and can be used as effective therapy for postprandial hyperglycemia with minimal side effects (Kwon et al., 2006, 2007). Furthermore, Kar et al. (2003) showed that crude ethanolic extract of snake tomato showed significant blood glucose lowering activity in alloxan induced diabetic albino rats and Arawwawala et al. (2009) using hot water extract of aerial parts of snake tomato also noted the improved glucose tolerance and tissue glycogen in non insulin dependent diabetes mellitus induced rats.

Conclusion

The inhibition of key enzymes linked with type-2 diabetes (α -amylase and α -glucosidase) and antioxidative properties of the "tomatoes" used in this study could make them good dietary means for the management and/or prevention of type-2 diabetes. The antioxidant properties of snake tomato which favourably compares with the other lycopersicon tomatoes (except CER), combined with its stronger inhibition of α -glucosidase activity, but milder inhibition of α -amylase activities suggests that snake tomato could be an alternative or complement to the use of lycopersicon tomatoes.

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