Liver Protective Activity of the Methanol Extract of Crinum jagus Bulb against Acetaminophen-induced Hepatic Damage in Wistar Rats

1Chinaka O. Nwaehujor, 2Florence C. Nwinyi and 3Julius O. Ode
1Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria
2Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Abuja, P.M.B. 117 Abuja, Nigeria

Corresponding Author: Chinaka O. Nwaehujor, Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria Tel: +2348035450300

ABSTRACT
Hepatotoxins constitute a serious health concern in both rural and urban population globally. Despite advances in medical research, the discovery of an ideal hepatoprotective agent remains a challenge. The present research sought to evaluate the hepatoprotective activity of the crude methanol extract of Crinum jagus bulb as a step towards further detailed study to isolate the bioactive principles. Wistar rats were pre-challenged individually with a high dose of acetaminophen (paracetamol, 2000 mg kg⁻¹) per os to induce hepatic damage prior to treatment. The control group was given distilled water (10 mL kg⁻¹, p.o.) while one out of the other experimental rat groups was either treated with silymarin (50 mg kg⁻¹, p.o.) or with a dose of C. jagus bulb extract (75, 150 and 300 mg kg⁻¹). Pentobarbitone-induced sleeping time, the mean relative liver weight of individual rats, biochemical assay and histopathological lesions in the liver of the separate rat groups were assessed and compared to determine the extent of hepatic damage. The prolonged paracetamol-induced pentobarbitone sleeping time in untreated, control rats (145.2±1.4 min) was most remarkably reduced to 122.5±2.1 and 109.5±0.4 min in rats which were treated orally with 150 and 300 mg kg⁻¹ of the extract respectively. The acetaminophen-mediated decrease in the mean relative liver weight of intoxicated rats was relatively reversed with 150 and 300 mg kg⁻¹ of the extract. C. jagus bulb extract also demonstrated significant (p<0.05) potency at 150 and 300 mg kg⁻¹ in reducing acetaminophen-induced increase in the rat serum transaminases (AST, ALT and ALP) and total bilirubin but with elevation in total serum protein values. Histopathology revealed that 2000 mg kg⁻¹ of paracetamol induced severe necrosis of hepatocytes in untreated control rats. Treatment of the acetaminophen-challenged rats with silymarin (50 mg kg⁻¹, p.o.) and C. jagus bulb extract (150 and 300 mg kg⁻¹, p.o.) gave a better protection with regeneration of hepatocytes relative to the untreated control. Crinum jagus bulb extract seemed to have multiplicity of effects in regenerating parenchymal cells, hepatic microsomal enzymes with high antioxidant and anti-inflammatory activities. The bulb of C. jagus could be a potential source of potent hepatoprotective agents.

Key words: Transaminases, hepatotoxicity, Crinum jagus, antioxidant, silymarin, acetaminophen

INTRODUCTION
Liver is a vital internal organ of the body and part of the digestive system (Karim et al., 2011). It is involved in first-pass effect which essentially, is concerned with the metabolism of orally
administered drugs by gastrointestinal and hepatic enzymes, resulting in a significant reduction of the amount of un-metabolized drug reaching systemic circulation (Kwan, 1997). Liver is also concerned with detoxification of drugs and food substances, deamination of excess proteins, storage of iron, vitamins and glycogen, production of bile, proteins and vital enzymes in the body. The strategic importance of the liver could be a reason for some of its naturally endowed qualities. Liver has a remarkable capacity to regenerate after injury and to adjust to size to match its host following transplant. Experiments have shown that within a week after partial heptectomy which involves surgical removal of two-thirds of the liver, hepatic mass regenerates back essentially to what it was prior to surgery (Michalopoulos and DeFrowces, 1997). Partial heptectomy reportedly leads to proliferation of all population of cells within liver, including hepatocytes, biliary epithelial cells and endothelial cells. DNA synthesis was noted to be initiated in these cells within 10 to 12 h after surgery and essentially ceases in about 3 days (Michalopoulos and DeFrowces, 1997).

The pivotal role of the liver in biotransformation however, makes it susceptible to toxic assault by xenobiotics (Craig and Stitzel, 1994). Liver could be damaged due to effects from medications e.g., acetaminophen, Paracetamol® (Bartlett, 2004), alcohol abuse (Bykov et al., 2004), hepatotoxins (Appiah et al., 2009), autoimmune hepatitis, viral and microbial infections (Ardanaz and Pagano, 2006). Fortunately, plants offer recipe for many health challenges. It is on record that a large section of the world’s population relies on herbal remedies to treat plethora of diseases due to their low costs, easy access and reduced side effects (Marino-Betilo, 1980) though pharmacological basis behind most herbal therapies however, remains practically unknown.

Plants are also a source of novel anti-oxidant and hepatoprotective agents since many industrial drugs are derived as a result of knowledge got from folklore medicine (Brand et al., 1991). Some plants with reported hepatoprotective properties are *Garcinia kola* Kor Gaul (Clusiaceae), *Tinospora cordifolia* (A. Rich.), *Ricinus communis* Linn. (Euphorbiaceae), *Curcuma longa* Linn. (Zingiberaceae), *Enicostemma liitorale* Blume (Gentianaceae), *Flaveria trinervia* Linn. (Asteraceae) and *Boerhaavia diffusa* Linn. (Nyctaginaceae) (Devaki et al., 2004; Umadevi et al., 2004; Vishwakarma and Goyal, 2004). Most of the herbal preparations speed up the natural healing processes of the liver (Senthilkumar et al., 2005). *Phellinus rimosus* (Berk) Platt (Hymenochaetaceae), a mushroom has been shown to protect the liver from acute and Chronic Carbon Tetrachloride (CCL4)-induced hepatotoxicity in rats by restoring the liver anti-oxidant status, inhibiting the phase I and enhancing the phase II enzyme activities (Ajith et al., 2006).

*C. jagus* commonly called Harmattan lily, belongs to Amaryllidaceae, a heterogenous family of 86 genera and about 1310 species (Lawrence, 1951). The plant is distributed worldwide in the tropics and subtropics. *C. jagus* is locally called ‘okonkilo inyi’ which literally means elephant’s potato by the Igede people of Benue State who inhabit the middle belt region of Nigeria. The plant is also called ‘gadali’ by the Hausa and Fulani tribes in Northern Nigeria (Dalziel, 1937). All the plant species are of ornamental value. In Sierra Leone, it is reported that a cold infusion of the fresh leaves is used to bathe young children suffering from general body debility, rickets, etc., (Dalziel, 1937). A decoction is given as a vermifuge in Gold coast (Ghana). The bulbs of several species are sold for various medicinal purposes in Lagos, Nigeria. In East Africa, the decoction of *C. jagus* is used for treatment of sores (Kokwaro, 1976). Ode and Asuzu (2006) reported that the methanol extract of *C. jagus* bulb exhibited antivenom effects when it completely inhibited the hemorrhagic activity of *Echis ocellatus* venom (4.2 µg 1.5 µL⁻¹) at various concentrations.
(2.5, 5.0 and 10.0 μg 1.5 μL⁻¹). The plant extract was also found to possess greater antioxidant activities at increased concentrations (50-400 μg mL⁻¹) compared to the reference non-enzymatic antioxidant (ascorbic acid) using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging spectrophotometric assay (Ode et al., 2010).

The present study was undertaken to evaluate the hepatoprotective effects of the methanol extract of C. jagus bulb against acetaminophen-challenged hepatic injury in rats.

MATERIALS AND METHODS

Chemicals, reagents and drugs: Ethanol and methanol (Riedel-De Haen AG-hanover), sodium chloride (BDH, England), silymarin (Legalon®, 70, Chemical Industries, Madaus AG, Germany), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), Ferric tripyridyltriazine (Fe (III)-TPTZ), Ascorbic acid and acetaminophen i.e., Paracetamol (Sigma Aldrich Sigma Aldrich, Germany), Pentobarbitone sodium (Abbot Laboratories Ltd., Kent, UK), Kits for Serum alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) (Randox Laboratories Ltd, United Kingdom), Total bilirubin and Total protein laboratory kits (Quimica Clinica Aplicada S.A., Spain) were used in the study.

Animals: Adult Wistar rats (130-180 g) obtained from the Laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka were used for the study. Animals were kept in stainless steel cages and had access to feed (Vital feed®, Nigeria Ltd.) and water ad libitum except in situations where fasting was required. The rats were allowed 14 days to acclimatize before the experiments were conducted according to the permission and prescribed guidelines of the Institutional Animal Ethics Committee. A total number of 36 Wistar rats were used for the experiments.

Plant materials and extraction: Fresh bulbs of C. jagus were collected in August, 2011 from farm locations in Ochimode Village, Oju Local government Area of Benue State, Nigeria. The plant materials were duly identified by Mr. A.O. Ozioko of the Department of Botany, University of Nigeria, Nsukka. C. jagus bulbs (CJJB) were dismembered and sliced into small pieces. They were air-dried for several weeks before being pulverized into coarse powder using hammer mill. One kilogram of the powdered bulbs was extracted by cold maceration with 80% methanol and intermittent vigorous shaking for 72 h. Concentration of the extract in vacuo with rotary evaporator afforded 12.6% w/w dry matter.

Effects of C. jagus bulb extract on acetaminophen-induced hepatotoxicity in rats: A total of thirty-six Wistar rats (130-180 g) were randomly allocated to 6 groups comprising of 6 animals in a group. All the rats except group 1 (normal) were fasted overnight and pretreated orally with a single dose (2000 mg kg⁻¹) of acetaminophen. Following 12 h after challenge with acetaminophen, group 2 (positive control) received distilled water (10 mL kg⁻¹, p.o.) only for 4 days. Group 3 rats were given silymarin (50 mg kg⁻¹, p.o.) for 4 days and this served as the reference drug for comparison. Groups 4-6 acetaminophen-challenged rats were treated orally with 75, 150 and 300 mg kg⁻¹ of the extract of C. jagus bulb respectively for 4 days. All treatments were given by stomach intubation. Pentobarbitone-induced sleeping time assay was carried out on day 4 by
intraperitoneal administration of pentobarbitone sodium (35 mg kg⁻¹). The sleeping time was calculated as the interval between the loss and recovery of the righting reflex (Shetty and Anika, 1982).

On recovery, blood samples were collected and the rats were humanely sacrificed. The blood samples were allowed to clot at room temperature. They were then centrifuged at 2,500 rpm for 10 min to separate the serum which was used to determine the serum ALT, AST, ALP, total bilirubin and total protein levels. All the rat livers were weighed for determination of the relative liver weight for each group. Tissue samples of the liver from each experimental group was collected and processed for comparison of histopathologic lesions.

**Biochemical assay:** The serum levels of ALT, AST were determined using the method of Reitman and Frankel (1957), serum ALP level was assayed by the method of King and King (1954), total bilirubin level by the method of Malloy and Evelyn (1937) as modified by Tietz (1996) and total serum protein level by the method of Johnson (1943).

**Relative liver weight:** Excess water from each of the rat liver samples was absorbed with a piece of serviette before weights were taken on a weighing balance (Metler, England). The individual rat liver weight was expressed in relation to the total body weight to obtain the relative liver weight. The total and the mean relative liver weight for each experimental group were determined and compared.

**Histopathology:** Tissue sample from the liver of rats in each group (1-6) of the experiment was fixed in 10% formal saline for a minimum of 24 h and then dehydrated by washing in ascending grades of ethanol before clearing with xylene and embedding in paraffin wax. The samples were sectioned with a microtome, stained with Hematoxylin and Eosin (H and E) and mounted on Canada balsam. All sections were examined under light microscope (10, 20 and x40) magnification. Photographs of the lesions were taken with an Olympus photo microscope for observation and comparison of histopathologic lesions.

**Statistical analysis:** All data collected were subjected to one-way Analysis of Variance (ANOVA) and Duncan’s New Multiple Range Test (DNMRT) was used as the post hoc test to separate the treatment means. Differences at p<0.05 were considered significant.

**RESULTS**

**Pentobarbitone-induced sleeping time:** The pentobarbitone-induced sleeping time was significantly (p<0.05) prolonged in acetaminophen-challenged, untreated rats (145.2±1.4 min) compared to the normal rats, Group 1 (85.2±0.6 min). The effect of the extract of *C. jagus* bulb at 75 mg kg⁻¹ on the pentobarbitone-induced sleeping time was not significantly (p>0.05) different from that of the control, which were challenged with acetaminophen (2000 mg kg⁻¹, p.o.) without treatment. However, silymarin (50 mg kg⁻¹, p.o.) gave 114.2±0.4 min while the extract gave 122.5±2.1 and 109.5±0.4 min at 150 and 300 mg kg⁻¹, p.o., respectively. The methanol extract of *C. jagus* bulb was therefore able to produce significant (p<0.05) decrease in pentobarbitone-induced sleeping time at 150 and 300 mg kg⁻¹ when compared with the mean sleeping time value in acetaminophen-challenged, untreated rats (Fig. 1).

The mean relative liver weights of acetaminophen-challenged rats: The mean relative liver weight became significantly (p<0.05) reduced in untreated, acetaminophen-intoxicated rats that were given only paracetamol (2000 mg kg⁻¹, p.o.) and also in rats that were treated with 75 mg kg⁻¹ of C. jagus bulb extract in contrast to the other groups (normal, silymarin, C. jagus bulb extract at 150 and 300 mg kg⁻¹). The mean relative liver weight of the normal rats was 30.5×10⁻²±1.8 g but untreated paracetamol-intoxicated rats had 20.8×10⁻²±1.3 g while rats that were treated with silymarin (50 mg kg⁻¹) had 27.8×10⁻²±2.6 g; 150 and 300 mg kg⁻¹ of C. jagus bulb extract produced 28.2×10⁻²±1.4 and 29.1×10⁻³±0.5 g, respectively (Fig. 2). There was no significant (p>0.05) difference between the mean relative liver weight of the normal rats and those of intoxicated rats that were treated with 150 and 300 mg kg⁻¹ of the crude extract of C. jagus bulb.

Acetaminophen-induced liver toxicity: The serum AST, ALT and ALP levels were significantly (p<0.01) increased in acetaminophen-challenged, untreated rats (136.8±3.6, 164.6±4.5, 54.0±7.6 μL⁻¹) respectively when compared to non-challenged normal group (54.6±1.4, 73.5±1.7, 25.2±1.4 μL⁻¹). AST value (136.8±3.6 μL⁻¹) and the total bilirubin (2.28±0.02 mg dL⁻¹) in the control, untreated rats were significantly (p<0.05) reduced to 118.9±1.3 μL⁻¹ and

![Fig. 1: The effect of methanol extract of C. jagus bulb and Silymarin on sleeping time in rats intoxicated with acetaminophen, Parac: Paracetamol, CJE: Crinum jagus bulb extract](image1)

![Fig. 2: The effect of the crude methanol extract of C. jagus bulb and silymarin on relative liver weights in acetaminophen-challenged rats, Parac, Paracetamol, CJE: Crinum jagus bulb extract](image2)
1.55±0.2 mg dL⁻¹ respectively following treatment with 75 mg kg⁻¹, p.o. of the extract but the protein level became significantly (p<0.05) increased comparable to the effects of the other doses (150 and 300 mg kg⁻¹) of the extract. The low dose (75 mg kg⁻¹) of the extract did not however, induce any significant (p>0.05) alteration on the serum levels of ALT and ALP in acetaminophen-challenged rats. The bulk extract of *C. jagus* (150 and 300 mg kg⁻¹, p.o.) significantly (p<0.05) decreased the elevated levels of AST, ALT and ALP compared to the acetaminophen-challenged untreated group. The reduction produced by the methanol extract of *C. jagus* on ALT, AST and ALP was highest at 300 mg kg⁻¹. Similarly, silymarin (50 mg kg⁻¹) and *C. jagus* extract (150 and 300 mg kg⁻¹) caused a significant (p<0.05) reduction in the total serum bilirubin level but a significant (p<0.05) increase in the total protein level of test rats when compared to acetaminophen-challenged, untreated group (Table 1).

Histopathology of paracetamol-induced hepatotoxicity in rats:

**Group 1:** Normal rats, no acetaminophen intoxication: The rat hepatocytes appeared normal without visible damage to the liver cells. Hepatocytes were seen typically radiating from the central vein; the stellate macrophage lined the endothelial cells. Some of the hepatocytes were mononucleated while others were binucleated (Fig. 3).

**Group 2:** Control, given distilled water 10 mL kg⁻¹, p.o.: There was severe necrosis and vacuolation of hepatocytes. The normal histological arrangement of hepatocytes in hepatic lobules and around the sinusoids was greatly distorted (Fig. 4).

**Group 3:** Acetaminophen-challenged rats treated with silymarin, 50 mg kg⁻¹, p.o.: There was serious regeneration and repair in response to the injurious effects of acetaminophen (2000 mg kg⁻¹, p.o.) as evidenced from the presence of megalocytes (mitotic cells) in the hepatic tissue (Fig. 5).

**Group 4:** Acetaminophen-challenged rats treated orally with 75 mg kg⁻¹ of *C. jagus* extract: There was mild coagulative necrosis even though some normal hepatocytes could be seen (Fig. 6). The liver damage caused a general disorganization of hepatocytes.

**Group 5:** Acetaminophen-challenged rats treated orally with 150 mg kg⁻¹ of *C. jagus* extract: Megalocytes were present showing the possibility of tissue repair taking place and some of the hepatocytes appeared normal (Fig. 7).

**Group 6:** Acetaminophen-challenged rats treated orally with 300 mg kg⁻¹ of *C. jagus* extract: There was dissemination of a large number of megalocytes, an indication of maximal regeneration within the hepatic tissue (Fig. 8).
Fig. 3: Normal rat liver, no acetaminophen toxicity, H and E x400, K: Normal hepatocytes

Fig. 4: Micrograph of the liver from acetaminophen, (2000 mg kg⁻¹) challenged rat, N: Severe necrosis of hepatocytes, H and E x400

Fig. 5: Micrograph of the liver from acetaminophen-challenged rat treated with silymarin (50 mg kg⁻¹, p.o.), Arrows point to megalocytes, evidence of regeneration taking place, H and E x400
Fig. 6: A liver section from acetaminophen-challenged rat treated with *C. jagus* extract (75 mg kg\(^{-1}\), p.o.), N: Necrotic hepatocytes, H: Normal hepatocytes, H and E x400

Fig. 7: Liver section of acetaminophen-challenged rat treated with *C. jagus* extract (150 mg kg\(^{-1}\), p.o.), M: Megalocytes, H: Normal hepatocyte, H and E x400

Fig. 8: Micrograph of the liver of acetaminophen-challenged rat treated with *C. jagus* extract (300 mg kg\(^{-1}\), p.o.), M: Megalocytes
DISCUSSION

The prolonged pentobarbitone-induced sleeping time observed in the paracetamol-challenged, untreated rats was found to be significantly (p<0.05) decreased when challenged rats were treated with the extract of C. jagus bulb (150 and 300 mg kg⁻¹). This could be possible due to the protective effects of the extract on the liver resulting in reduced destruction of hepatocytes which remained viable to carry out biotransformation of the drug. The anaesthetic, pentobarbitone sodium was metabolized by active liver cells and this culminated in the reduced duration of anesthesia in the treated rats. Again, the paracetamol-intoxicated, untreated control and rats that were treated with low dose (75 mg kg⁻¹) of C. jagus bulb extract had significantly (p<0.05) reduced mean relative liver weight compared to others (normal rats and groups that were treated with silymarin and the extract at 150 and 300 mg kg⁻¹). The reduction in the mean relative liver weight could be as a result of acute necrosis of hepatocytes in the absence of adequate hepatoprotective agent. Liver is responsible for biotransformation of toxic substances, including drugs and hormones (Banks, 1993). Increasingly large numbers of drugs, herbicides, food additives and environmental carcinogenic hydrocarbons are found to stimulate their own metabolism or the metabolism of other compounds by increasing the amount of drug metabolizing enzymes in liver microsomes (Conney, 1967). The crude extract of C. jagus bulb could have aided the rejuvenation of hepatic microsomal enzymes which subsequently carried out accelerated metabolism of pentobarbitone sodium with resultant decrease in duration and intensity of the anaesthetic effect. The extract may contain or stimulate chemical mediators in the local tissue microenvironment to enhance cell growth. Polypeptide growth factors stimulate cellular proliferation and also mediate a wide variety of other activities, including cell migration, differentiation and tissue remodeling (Kumar et al., 1997). Crinum jagus bulb extract may have aided liver parenchymal cell growth in acetaminophen-intoxicated rats at 150 and 300 mg kg⁻¹. Cell growth factors are involved in various stages of wound healing.

The methanol extract of C. jagus bulb was also effective at 150 and 300 mg kg⁻¹, p.o. in reducing the serum levels of AST, ALT and ALP and also preserved the functional ability of the liver. This was revealed when both doses of the extract produced significant (p<0.05) reduction in the level of transaminases and the total bilirubin but increased total protein levels relative to acetaminophen-challenged, untreated rats. The conjugating and synthesizing ability of the liver was therefore intact. The extract did show minimal hepatoprotective activity at 75 mg kg⁻¹ when there was significant (p<0.05) decrease in the mean serum AST and total bilirubin levels but elevated total protein values relative to the control, untreated rats. The serum concentration of ALT and ALP, however, remained high post treatment with 75 mg kg⁻¹, p.o. of the extract which was suggestive of a persisting hepatic tissue injury. Hepatic cells contain higher concentrations of AST and ALT in the cytoplasm but AST in particular exists in the mitochondria (Wells, 1988). Damage to hepatic cells induces leakage into plasma leading to an increased level of hepato-specific enzymes in serum (Tolman and Rej, 1999). The measurement of serum AST, ALT and ALP levels serve as a means for indirect assessment of liver function.

Toxic injury to hepatocytes stimulates inflammatory reactions. The cell membrane damage associated with inflammation results in leucocyte release of lysosomal enzymes that can be injurious to nearby cells (Konturek et al., 2000). Cell damage causes the release of arachidonic acid and pro-inflammatory cytokines. Stimulation of neutrophils can lead to the production of oxygen-derived free radicals that produce further cellular damage (Forman and Torres, 2002). Crinum jagus bulb was found to possess a significantly higher antioxidant activity compared to ascorbic acid (Ode et al., 2010). The mechanism of the hepatoprotective activity of the extract of C. jagus bulb
in acetaminophen-challenged rats may be derived from some anti-inflammatory and antioxidant principles in the extract. The extract could also have caused accelerated regeneration of damaged liver cells. Active regeneration of hepatocytes in the form of megalocytes (mitotic liver cells) was prominently seen in the tissue section of the liver of acetaminophen-challenged rats which were treated with 150 and 300 mg kg\textsuperscript{-1}, p.o. of the extract. Mitosis precedes regeneration and replacement of worn-out cells. During the cell division, each chromosome made up of two chromatids attach randomly at the centromere and later split. Daughter chromosomes begin to migrate to the opposite poles in anaphase but in telophase, nuclear reconstitution and enlargement occurs; cytokinesis ensues resulting in two identical daughter cells (Banks, 1993). Cell proliferation from mitosis is vital for tissue repair.

*C. jagus* bulb extract could have aided regeneration of parenchymal cells and hepatic microsomal enzymes at 150 and 300 mg kg\textsuperscript{-1}. The hepatoprotective effect may also be due to the high antioxidant activity and some anti-inflammatory principles in the extract.

CONCLUSION

The methanol extract of *C. jagus* bulb (150 and 300 mg kg\textsuperscript{-1}, p.o.) and silymarin (50 mg kg\textsuperscript{-1}) demonstrated appreciable potency at reducing serum levels of AST, ALT, ALP and total bilirubin but increased total serum protein level in acetaminophen-induced liver damaged rats which was suggestive of liver protection. The hepatoprotective activity of the plant extract was also exhibited when the prolonged pentobarbitone-induced sleeping time in the acetaminophen-challenged rats became significantly reduced relative to the control, untreated rats. The mean relative liver weight of intoxicated rats which were treated with 150 and 300 mg kg\textsuperscript{-1} of the extract became comparable to the normal. The histopathology of the liver tissue from the experimental animals showed signs of regeneration and more protected hepatocytes in the *C. jagus* bulb extract-treated rats. Further studies to isolate the hepatoprotective principles in *C. jagus* bulb and to determine the mechanism of action are highly recommended.

REFERENCES


