EFFECTS OF ESCULETIN AND SCOPOLETIN ON TOTAL AND CONJUGATED BILIRUBIN LEVEL IN PARACETAMOL-INDUCED LIVER DAMAGE IN RATS

[1]Oruma, Y.U., [2]Abraham, O.J. and [3]Okutachi, A.M.
[1,2]Deptartment of Science Laboratory Technology, Federal Polytechnic, Idah, Kogi State, Nigeria
[3] Department of Mathematics and Statistics, Federal Polytechnic, Idah, Kogi State, Nigeria
Correspondence: uneksmusa79@gmail.com

ABSTRACT

This study determined the total and Conjugated Bilirubin in Esculetin and Scopoletin treated paracetamol-induced liver damage in rats. Thirty-five Male Wistar albino rats were divided into five groups of seven rats each. Two groups were administered 6mg/kg (as single dose) body weight of esculetin and scopoletin respectively daily for 28 days. Of paracetamol 500mg/kg body weight of paracetamol on the 28th day. A third group received single dose of 500mg/kg body weight on the 28th day. The fourth and fifth groups received 10% Dimethyl sulfoxide (DMSO) and distilled water respectively for 28 days. The rats were bled 48 hours after exposure to paracetamol. The mean concentration of conjugated bilirubin in the esculetin and scopoletin groups were 0.48 mg/ml and 0.31 mg/ml respectively, while that of the groups that received paracetamol, distilled water and diluents were 0.55 mg/ml, 0.32 mg/ml and 0.30 mg/ml respectively. The mean total bilirubin in the groups that received esculetin and scopoletin were 0.47 mg/ml and 0.30 mg/ml respectively, while that of the groups that received paracetamol, Diluent and distilled water only were 0.56 mg/ml, 0.32 mg/ml and 0.28 mg/ml respectively. A significant difference (P<0.05) was observed between the positive and negative control. There was no significant difference (P>0.05) between the effects of esculetin and scopoletin on the bilirubin level. However, significant difference in the level of conjugated and total bilirubin between the positive control and the test groups administered esculetin and scopoletin was observed.

Keywords: Esculetin, Scopoletin, Bilirubin, Paracetamol- induced liver damage, Wistar Albino Rat

INTRODUCTION

Natural products are typically secondary metabolites produced by organisms in response to external stimuli such as nutritional changes, infection and competition (Ojala, 2001). Natural products produced by plants, fungi, bacteria, insects and animals have been isolated as biologically active pharmacophores. Approximately, one-third of the top-selling drugs in the world are natural products or their derivatives often with ethnopharmacological background (Ojala, 2001). Moreover, natural products are widely recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities (Matos *et al*., 2015).

Secondary metabolites in natural products chemistry are considered as waste products of primary metabolic processes (biosynthesis, biodegradation and other energy conversions of intermediary metabolism) in biological systems (Arogba, 2008).

Plants are able to synthesize and accumulate a vast array of primary and secondary chemicals useful for the plant itself for protection against environmental stress factors due to their specialized biochemical capabilities. These compounds have made many plants useful also for humans for instance as spices, medicines, etc (Kostova, 2005).

Esculetin also known as 6, 7-dihydroxycoumarin is a phenolic antioxidant compound found in cassava (*Manihot esculenta crantz*), *Cichorium intybus* and *Bougainvillea spectabillis* (Gilani *et al.*, 1998; Abarca-Vargas and Petricevich, 2018). Esculetin can also be isolated from many plants such as *Artemisia spp., Citrus limona* and *Euphobia lathyns* (Leung *et al.*, 2005). It appears in its physical state as a yellow crystalline powder. It is slightly soluble in water

and weakly acidic. Its melting point is between 265–270^{o}C. Esculetin is stable under normal conditions. It is a 7-hydroxycoumarin and can be used to absorb ultraviolet rays in sunscreen cosmetics and also used in the synthesis of drugs especially anticancer drugs (Kostova, 2005). Esculetin protects cells from injury by linoleic acid hydroperoxide in cell incubated in medium supplemented with linoleic acid hydroperoxide (Kaneko *et al.*, 2003; Egbuonu *et al*., 2015)).

Scopoletin (6-methoxy -7- hydroxy coumarin) is a yellow to beige crystalline powder found in Noni, manaca, passion flower, cotton leaf, citrus peel, cassava (*Manihot esculenta*) and stevia as a fluorescent coumarin compound (Abdulrahman *et al.*, 2005). It is one of the most widespread coumarins in nature (Basaran *et al.*, 2007). It is used as a dye to detect the release of reactive oxygen species during the oxidative burst, peroxynitrite scavenger and as acetylcholinesterase inhibitor (Obidoa and Obasi, 1991; Ezeanyika *et al.*, 1999). Scopoletin has bacteriostatic activity against various species of bacteria including *Escheria coli, Staphylococcus aureus, Streptococcus spp, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Jain and Joshi, 2012). Its anti-inflammatory activity makes it a useful compound in the treatment of bronchial illness and asthma (Kostova, 2005) and in the regulation of the hormone serotonin which helps to reduce anxiety and depression (Albert *et al*., 2014; Kirsch *et al*., 2016). Scopoletin also relaxed the smooth muscles and inhibited the spasmogenic activities of a wide variety of agonists on guinea pig isolated ileum (Ojewole and Adesina, 1983). Scopoletin inhibited proliferation of certain cancer cells by inducing apoptosis (Liu *et al.*, 2001).

Paracetamol or acetaminophen is a common analgesic and antipyretic drug that is used for the relief of fever, headaches and other minor aches and pains (Janbaz and Gilani, 2002). Acetaminophen is the active metabolite of phenacetin responsible for its analgesic effect (Venkatesha and Kala, 2000). It is a weak prostaglandin inhibitor in peripheral tissues and possesses no significant anti-inflammatory effects (Betram, 2001), hence used for the treatment of mild to moderate pain when an anti-inflammatory effect is not necessary (Nwachukwu, 2006).

The liver plays a major role in metabolism, undertaking thousands of complex biochemical interactions that influence all the organs in the body. The liver has as many as 500 vital functions. It is involved in glycogen storage, decomposition of red blood cells, plasma protein synthesis, detoxification and production of bile. It also performs and regulates a wide variety of high-volume biochemical reactions requiring very specialized tissues (Boyer, 2013).

Bilirubin (serum bilirubin, urine bilirubin and urobilirubin)is an endogenous anion derived from haemoglobin degradation from the red blood cell (RBC) and is used to test the liver's capacity to transport organic anions and to metabolise drugs (Levitt and Levitt, 2014; Cvorovic´ and Passamonti, 2017).

Herbs have been used as food and for medicinal purposes for centuries, and in different herbs, a wide variety of active phytochemicals including coumarins have been identified. Many of the herbs contain potent anti-oxidant compounds that provide significant protection against chronic diseases. The liver is the largest organ in the body and serves as the site for xenobiotic metabolism and a lot of other biochemical activities. It is central to life and hence should be protected. Exposure of the liver to certain toxins results in liver damage and eventual death.

Paracetamol is a pain relieving drug and can be assessed by mainly everybody. Abuse of this drug has detrimental effect on the liver. Esculetin has been studied and shown to have possible protective effect against paracetamol induced liver damage (Gilani *et al.*, 1998). This study therefore compared the effects of esculetin and scopoletin on the level of Total Bilirubin (TB) and Conjugate Bilirubin (CB) in paracetamol- induced liver damage in male Wistar rats.

MATERIALS AND METHODS

Experimental Animals and Sources

Male Wistar albino rats of between four to eight weeks old with average weight ranging from 150 –230g were obtained from the Animal houses of Faculty of Biological Sciences and Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria.

Animal Care and Management

Thirty-five male Wistar rats were housed in separate cages, acclimatized for seven days and then divided into five groups of seven each. The animals were maintained on a twelve hour light and dark cycle under tropical conditions. They had free access to water and were fed with normal rat diet *ad libitum*. The normal rat diet was purchased from Bendel Feed and Flour Mills Limited., Ewu, Edo State, Nigeria.

Experimental Design

Animals in group-I were administered 6mg/kg (as single dose) body weight of esculetin by gastric intubation daily for 28 days. Animals in group II were administered 6mg/kg body weight of scopoletin by gastric intubation. The animals in the two groups were administered 500mg/kg body weight of paracetamol on the 28th day. Animals in group III were administered single dose of 500mg/kg body weight) of paracetamol by gastric intubation on the 28the day. This represented positive control. Animals in group IV were administered 10% DMSO for 28 days representing diluent control. Animals in group V administered distilled water only for 28 days representing negative control. The rats were weighed at the beginning of the experiment in order to predetermine dosage. After exposure to the toxicant (paracetamol) on the 28th day, the rats were allowed to survive for 48 hours and bled by ocular puncture before they were sacrificed.

Preparation of 6mg/kg Body Weight of Scopoletin Solution

Scopoletin solution was prepared by dissolving 36mg of scopoletin in 6ml of 10% DMSO.

Preparation of 6mg/kg Body Weight Esculetin Solution

This was prepared by dissolving 36mg esculetin in 6ml of 10% DMSO.

Preparation of 500mg/kg Body Weight Paracetamol Solution

Paracetamol (3125.0mg) was dissolved in 50ml of distilled water.

Preparation of Serum Samples

Whole blood was collected from the animals in different groups through ocular puncture into clean non-anticoagulated blood sample containers and centrifuged at a speed of 3000 rpm to obtain supernatants (serum). The sera gotten were used immediately for biochemical analyses.

Assay of Bilirubin

A colorimetric method with a kit supplied by Randox® was used as described by Jendrassik and Grof (1938) and Sherlock (1951).

Principle

Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid.

Reagent composition

	Contents	Initial concentration of solution
1.	Sulphanilic acid	29mmol/L
	Hydrochloric acid	0.17N
2.	Sodium Nitrate	25mmol/L
3.	Caffeine	0.26mol/L
	Sodium benzoate	0.52mol/L
4.	Tartrate	0.93mol/L
	Sodium hydroxide	1.9N

Procedure

Total Bilirubin (TB)

Reagent-1 (sulphanilicacid, hydrochloric acid), 0.20ml, was pipetted into two different cuvettes labeled sample blank (B) and sample (A) respectively, then a drop (0.05ml) of reagent was introduced. Then a drop of 0.05ml of reagent was pipetted into the cuvette containing sample (A) only.

Afterwards, 1.0ml of reagent 3 (caffeine, sodium benzoate) was pipetted into the cuvettes containing samples B and A respectively. Serum sample (0.2ml) was then pipetted into both cuvettes, sample blank (B) and sample (A). Their contents

were separately mixed and allowed to stand for 10min at 25°C.

This was followed by addition of 1ml of reagent 4 (Tartrate, sodium hydroxide) into both cuvettes containing sample blank and sample. They were mixed and allowed to stand for 30 minutes at 25°C. Finally, absorbance of the sample against the sample blank (A_{TB}) was read at 560nm. Total bilirubin values were obtained using the calculation below:

- Total bilirubin (µmol/L) = 185 x A_{TB} (560nm).
- Total bilirubin (mg/dl) = 10.8 x A_{TB} (560nm)

Direct Bilirubin (DB)

Reagent 1 (0.2ml) was pipetted into two cuvettes labeled sample blank (B) and sample (A). A drop (0.05ml) of reagent 2 was pipetted into the cuvette containing sample (A) only. This was followed by the addition of 2.0ml sodium chloride (µg/L) into both cuvettes containing sample blank (B) and sample (A) respectively. Serum (0.2ml) was finally pipetted into both cuvettes, sample blank (B) and sample (A). The contents of the two cuvettes were mixed thoroughly and allowed to stand for exactly 5min at 25°C. The absorbance of the sample against the sample blank (A_{DB}) was read at 550nm.

Direct bilirubin values were obtained from the following calculation:

Direct bilirubin (µmol/L) = 246 x A_{DB} (550nm)

Direct bilirubin (mg/dl) = 14.4 x A_{DB} (550nm)

Normal value in serum

Total bilirubin	up to 17 µmol/L
	up to 1 mg/dL
Direct bilirubin	up to 4.3 µmol/L
	up to 0.25 mg/dL

Statistical analysis

Test of statistical significance was carried out using one-way ANOVA with postHoc test (Multiple comparisons). Statistical difference was considered when P≥0.05.The Statistical Package for Social Sciences (SPSS) was used.

RESULTS

Comparative Effects of Esculetin and Scopoletin on Conjugated Bilirubin

The mean concentration of conjugated bilirubin in the group that received esculetin and paracetamol was 0.48 mg/ml, while that of the group that received scopoletin and paracetamol was 0.31 mg/ml (Fig. 1). The mean conjugated bilirubin level in the group that received paracetamol only was 0.55 mg/ml, while that of Diluent control (10% DMSO) only was 0.32 mg/ml and the level for the group that received distilled water only as negative control was 0.30 mg/ml (Fig 1).

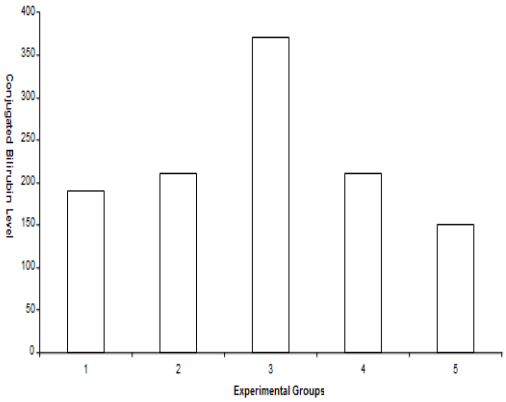

Fig. 1: Comparative effect of Esculetin and Scopoletin on the Conjugated Bilirubin level of Paracetamol-induced liver damage in rats

Comparative effects of esculetin and scopoletin on total bilirubin level

The mean total bilirubin concentration in the group that received esculetin and paracetamol was 0.47 mg/ml, while that of the group that received scopoletin and paracetamol was 0.30 mg/ml (Fig. 2).

The mean total bilirubin level in the group that received paracetamol only was 0.56 mg/ml, while that of Diluent control (10% DMSO) only was 0.32 mg/ml and the level for the group that received distilled water only as negative control was 0.28 mg/ml (Fig. 2).

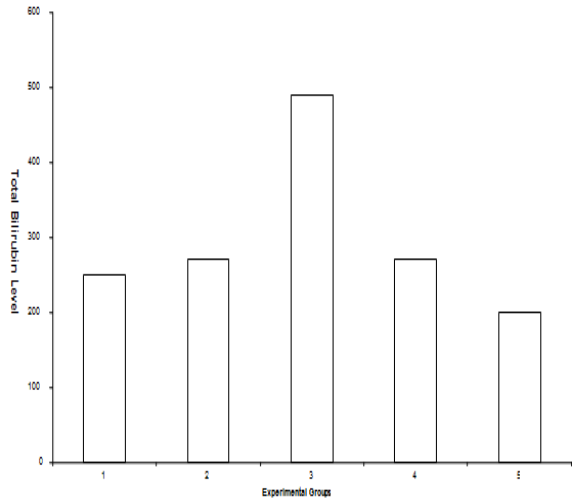

Fig. 2: Comparative effect of Esculetin and Scopoletin on the Total Bilirubin level of Paracetamol-induced liver damage in rats

Group 1 = Paracetamol + Esculetin

Group 2 = Paracetamol + Scopoletin

Group 3 = Paracetamol + DMSO

Group 4 = DMSO

Group 5 = Distilled Water

DISCUSSION

The results shown in fig.1 indicate that no significant difference (P>0.05) in conjugated bilirubin was observed between the test groups administered 6mg/kg body weight esculetin + 500mg/kg body weight of paracetamol and the group administered 6mg/kg body weight scopoletin + 500mg/kg body weight of paracetamol respectively. A significant difference (P<0.05) in conjugated bilirubin was observed between the positive control and the negative control given distilled water. No significant difference (P>0.05) was also observed between the test groups administered esculetin + paracetamol and scopoletin + paracetamol and the negative control group. There was a significant difference (P<0.05) observed between the test groups and negative control group. The results also showed that there was no significant difference in the level of conjugated bilirubin between the positive control and the test groups administered 6mg/kg body weight esculetin + 500mg/kg body weight of paracetamol respectively. Significant difference (P<0.05) was observed between the positive group administered scopoletin 6mg/kg body weight + 500mg/kg body weight of paracetamol respectively. There was also no significant difference (P>0.05) observed between the positive control and the group administered 10% DMSO.

Significant difference (P<0.05) was observed between the total bilirubin level of the test groups administered 6mg/kg body weight esculetin + 500mg/kg body weight of paracetamol and scopoletin 6mg/kg body weight + 500mg/kg body weight of paracetamol. No significant difference (P>0.05) was observed between the total bilirubin level of the esculetin + paracetamol group and the positive control. Also a significant difference (P<0.05) was observed between the total bilirubin level of scopoletin + paracetamol group and the positive control. A significant difference was observed between the test group, esculetin + paracetamol.

The liver is an organ of paramount importance which plays an essential role in the metabolism of foreign compounds entering the body. Human beings are exposed to these compounds through environmental exposure, consumption of contaminated food or during exposure to chemical substances in the occupational environment. In addition, human beings consume a lot of synthetic drugs during disease conditions which are alien to the body. All these compounds produce a variety of toxic manifestations (Atha *et al.*, 1997).

Paracetamol induced hepatic injuries are commonly used models for screening hepatoprotective activity of substances and the extent of hepatic damage is assessed by the level of increase in cytoplasmic ezymes (ALP, AST and ALT) in circulation (Janbaz and Gilani, 2002).

Esculetin is a phenolic antioxidant compound as reported by Gilani *et al*. (1998). It has also been shown by Gilani *et al*., (1998), to have a protective effect on paracetamol- induced liver damage. Oral administration of paracetamol produced liver damage in rats. Pretreatment of rats with esculetin prevented the paracetamol-induced rise in serum enzymes indicating that esculetin possesses anti-hepatic activity due to its antioxidant nature. Scopoletin is also an antioxidant coumarin since it possesses an hydroxyl group in its structure and thereby has the tendency of reversing damage to liver. Antioxidants are compounds capable of slowing or preventing the oxidation of other

molecules. Antioxidants are often reducing agents such as thiols or poly phenols. They act as a major defence against radical-mediated toxicity by protecting the damages caused by free radicals (Egbuonu *et al*., 2015).

No significant difference (P>0.05) was observed between the groups administered 6mg/kg body weight esculetin and 6mg/kg body weight scopoletin for all parameters assayed. As shown by this result, comparing the effect of esculetin and scopoletin on paracetamol- induced liver damage in rats, no significant difference was observed in their effect and none among the two can be said to be more effective. This is possibly due to the fact that both esculetin and scopoletin have their OH groups on the ortho positions and are equally phenolic. Also as reported by Kaneko *et al.* (2003), catechilic coumarins have a protective effect against cell damage. They reported that esculetin protected cells from injury by linoleic acid hydroperoxide in cells incubated with acid hydroperoxide. This is in tandem with this work since esculetin and scopoletin are catechilic coumarins.

CONCLUSION

This work suggests that paracetamol is a potential toxicant which induced liver intoxication. Esculetin and scopoletin were observed to have potential efficacy in protecting against paracetamol induced liver damage in rats. The pre-treatment of rats with esculetin and scopoletin for 28 days before exposure to the toxicant (paracetamol) significantly reduced the increased levels of liver enzyme markers in the blood, especially total bilirubin level, thus supporting the reports that esculetin and scopoletin protect against paracetamol-induced liver damage. Conscious and unconscious exposure to different potential toxicants should be avoided. Esculetin and scopoletin, therefore, possess hepatoprotective activity.

REFERENCES

Abarca-Vargas, R. and Petricevich, V.L. (2018). *Bougainvillea* Genus: A Review on Phytochemistry, Pharmacology, and Toxicology. Evidence-Based Complementary and Alternative Medicine. Volume 2018, Article ID 9070927, 17 pages. https://doi.org/10.1155/2018/9070927.

Abdulrahman, M., Omar, A.I. and Al-Arifi, M.N. (2005). Comparative Effects of Scopoletin and Menadione on Aldehyde Oxidase Activity of Guinea Pig Liver. *J. Biol Sc***.** 40:1-6.

Albert, P.R. , Vahid-Ansari, F. and Luckhart, C. (2014). Serotonin-prefrontal cortical circuitry in anxiety anddepression phenotypes: pivotal role of pre-andpost-synaptic 5-HT1A receptor expression. *Frontiers in Behavioral Neuroscience.* 8(199): 1-13

Arogba, S.S. (2008). Phenolics: *A Class of Natures Chemical Weapons of Self Preservation*. 1st Inaugural Lecture, Kogi State University, Anyigba: Kogi State.

Athar, M., Zakir, H. S. and Hassan, N.(1997). Drug metabolizing enzymes in the liver. Liver and Environmental Xenobiotics. New Delhi: Narosa Publishing House. Available on line at http:// www.ijp-online.com/article.asp?issn=0253-7613,year=2004; volume=36;issue=5.

Basaran, I., Sinan, S., Cakit, U., Buluf, M., Arslan, O. and Ozensory, O. (2007). *In vitro* Inhibitor of Cytosolic Carbonic Anhydrase I and II by some New dihydroxycoumarin Compounds. *J. Enzy. Inhib. And Med. Chem*. **23**(1): 32-36.

Betram, G.K. (2001). *Basic and Clinical Pharmacology*. 8th edition. McGraw Hill: USA. p. 615.

Boyer, J.L. (2013): Bile Formation and Secretion. *Compr. Physiol*. 3(3): 1035–1078. doi:10.1002/cphy.c120027.

Cvorovic,´ J. and Passamonti, S. (2017). Membrane Transporters for Bilirubin and Its Conjugates: A Systematic Review. Front. Pharmacol. 8 (887): 1-17 doi: 10.3389/fphar.2017.00887

Egbuonu**,** A.C. C**.,** Ogbu**,** A.E**.,** Ijeh**,** I.I. and Ezeanyika, L.U.S. (2015)**.** Sub-Chronic Esculetin (6,7-Dihydroxy-Coumarin)-Induced Alteration in Some Haematological and Serum Parameters in Normal Male Wistar Rats . *Asian Journal of Biochemistry*. 10 (6): 306-311.

Ezeanyika, L.U.S., Obidoa, O. and Shoyinka, V.O. (1999). Comparative Effects of Scopoletin and Cyanide on Rat Brain. 1. *Histopathology Plant Foods for Human Nutrition* 53(4):351-358.

Gilani, A.H., Janbaz, K.H. and Shah, B.H. (1998). Esculetin Prevents Liver Damage Induced by Paracetamol and CCl_4. *Pharm. Res*. 37(1):31-35.

Jain, P.K. and Joshi, H. (2012). Coumarin: Chemical and Pharmacological Profile. *Journal of Applied Pharmaceutical Science*. 2(06);:236-240.

Janbaz, K. H. and Gilani, A.H. (2002). Methol Prevents Liver Damage Induced by Paracetamol and CCl_4. *Pakistan J. Bio Sc*. 5(10): 1001-1103.

Kalakonda, A. and John, S. (2019). Physiology, Bilirubin. StatPearls [Internet]. Available at https://www.ncbi.nlm.nih.gov/books/NBK470290/. Accessed on 28/5/2019.

Kaneko, T., Baba, N. and Matsuo, M. (2003). Protection of Coumarins against Linoleic Acid Hydrogen peroxide-Induced Cytotoxity, *Chem. Biol. Interact* 142(3). 239-254.

Kirsch, G., Abdelwahab, A.B. and Chaimbault, P. (2016). Natural and Synthetic Coumarins with Effects on Inflammation. *Molecule*. 21, 1322; doi:10.3390/molecules21101322

Kostova, I. (2005). Synthetic and Natural Coumarin as Cytotoxic Agents. *Curr. Med. Chem. Anticancer Agents*. 5(1) 28-45.

Leung, K., Leung, P., Kong, L. and Leung, P. (2005). Immunomodu-latory Effects of 6,7-Dihydroxycoumarin on Marine Lymphocytes and Peritoneal Macrophages. *Chinese Society of Immunology* 2(3):181-186.

Levitt, D. G., and Levitt, M. D. (2014). Quantitative assessment of the multiple processes responsible for bilirubin homeostasis in health and disease. Clinical and Experimental Gastroenterology. 7 307–328.

Liu, X., Zhang, L.., Fu, X., Chen, K. and Qian, B. (2001). Effect of Scopoletin on PC3 Cell Proliferation and Apoptosis. *Acta Pharmacol*. 22(10):929-933.

Jendrasskik, J. and Grof. P. (1938). *In vitro* Determination of Total and Direct Bilirubin in Serum or Plasma. *Biochem*. 297: 81.

Matos , M. J. , Santana , L., Uriarte , E., Yordi , E.G., Abreu , O. and Pérez, E.M. (2015). Coumarins — An Important Class of Phytochemicals. In: Rao, V. (Editor): *Phytochemicals - Isolation, Characterisation and Role in Human Health.* DOI: 10.5772/59982. Publisher: InTech.

Nwachukwu, O. (2006). Hepatoprotective Activities of Coconut (*cocus nucifera*) Water in Paracetamol Intoxicated Rats. *A Research Project Presented to the Department of Biochemistry*, UNN.

Obidoa, O. and Obasi, S. C. (1991). Comparative Effects of Scopoletin and Cyanide on Rat Brain. *Plant Foods for Human Nutrition.* 41(3): 283-289.

Ojala, T. (2001). *Biological Screening of Plant Coumarins*. Academic Dissertation. Helsinki.

Ojewole, J.A.O. and Adesina, S.K. (1983). Evaluation of the Mechanism of the Hypotensive Effect of Scopoletin Isolated from the Fruit of *Tetrapleura tetraptera* Plant. *Planta Med*. 49:46-50.

Sherlock, S. (1951). *Liver Disease*. Churchill: London. p. 204.

Venkatesha, U. and Kala, S.K. (2000). Effects of HD-O3 on Levels of Various Enzymes in Paracetamol-Induced Liver Damage in Rats. *Indian J. Pharm.* 32:361-364.