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In Vitro Evaluation Of Hepatoprotective Activity Of Siddha Medicine – Veppam Poo Ooral Kudineer By Acetaminophen Induced HepG2 Cell Line

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KEYWORDS
Siddha medicine, Veppam poo ooral kudineer, Hepatotoxicity, Azadirachta indica (Neem), Hepatoprotective.

ABSTRACT:
Among the traditional medicines, Siddha medicine is becoming widely spread among south Indian population. In ancient period, the primary line of treatment for liver problems by Siddha drugs. There are several herbal prescriptions on the market. Azadirachta indica (Neem) was a commonest plant present all over the Tamil Nadu. The compound quercetin and rutin, Nimbolide in A.indica have hepatoprotective activity.Veppam poo ooral kudineer is one the Siddha preparation indicated for hepatoprotective in Siddha literature. The aim of the study to evaluvate the the hepatoprotective activity of Veppam poo ooral kudineer(VOK). VOK was prepared according to the method given in Siddha literature. Hepatoprotective activity was evaluated against Acetaminophen induced hepatotoxicity by its effect on the HepG2 cell line. The sample VOK increased significantly of HepG2 cells at around 84% in VOK at concentration 100 µg/mL compared to the normal cells.

Introduction:

Liver is considered a vital organ of the human body involved in important functions. It preserves the body against foreign particle by elimination through the excretion process. Some drugs for prolong period may cause hepatotoxicity. Many synthetic drugs currently available for the treatment of liver disease are inadequate and are known to have various side effects (1). Acetaminophen, also known as APAP (in the United States), paracetamol (in Europe and other areas of the world) or N-acetyl-p-aminophenol, is one of the most commonly utilized compounds worldwide (2). Acetaminophen is responsible for an estimated 48% of all acute liver failure diagnoses (3). Acetaminophen a well-known for Non-Steroidal Anti-inflammatory Drug (NSAID), anti-allergic and antipyretic drug. It is the over-the-counter drug. APAP overdose causes severe hepatotoxicity that leads to liver failure in both humans and experimental animals (4) .A small amount of APAP is metabolized together with cytochrome P450. As a result, N-acetylp-benzoquinone imine (NAPQI) or N-acetyl-p-benzosemiquinone imine (NAPSQI) appears in the body's system(5).In India, Drug-induced liver injury(DILI) as it occurs in acetaminophen/paracetamol hepatotoxicity accounts for <1% of cases(6). APAP hepatotoxicity occurs through formation of the noxious NAPQI metabolite, which is present in excessive quantities, as augmented by features of glutathione (GSH) depletion, oxidative stress and mitochondrial dysfunction leading to depletion in adenosine triphosphate (ATP) stores.(7)

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical d isease. More attention has been paid to the protective effects of natural antioxidants against drug-induced toxicities especially whenever free radical generation is involved. Flavonoids have been found to play important roles in the non-enzymatic protection against oxidative stress(8).

Azadirachta indica (Neem) was a commonest plant present all over the Tamil Nadu.

It belong to meliaceae family has several medicinal values. The leaves, flowers, seeds, roots and bark of the plant possess important active constituent is Azadirachtin, Nimbolinin, Nimbin, Nimbidin(9), Quercetin and ß-sitosterol(10).The compound Azadirachtin-A in A.indica have hepatoprotective activity(11) .

In Siddha literature, the flower of Azadirachta indica(veppam poo) ooral kudineer was indicated for

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hepato protective(12). Kudineer(Decotion) also named as Kiyazham, Kashayam, Unneer, Marundhu neer(13). The shelf life of this form of medicine is one saamam (three hours) and hence it should be consumed within the specified period from the time of preparation (14). There are two types of Kudineer viz. Ooral kudineeer (prepared after soaking the contents for 2-12 hours or overnight) and Kodhi Kudineer (prepared by boiling). The kudineer is used in external wash, gargling, drinking, wound cleaning, and purgation. Decoctions can be used both internally and externally according to the disease condition. As a whole, decoctions are water-based extracts of either herbal, herbo-mineral or animal-based ingredients which are easily absorbed into the body and enter into the blood stream rapidly for better efficacy (15).

Among them, Ooral Kudineer is one of the internal medicine, defined as dilute liquid extract obtained from a drug or drugs by soaking it in water (cold or hot) (16). Hence the present study was undertaken to evaluate the Hepato-protective Activity of Veppam poo ooral kudineer(VOK) by Acetaminophen induced cell line invitro method.

Material and Methods:

The Flower of Azadirachta indica(veppam poo) used in this study was collected during the month of April-May month from campus of National Institute of Siddha, Chennai and authenticated by Botanist, National Institute for Siddha, Chennai (NISMB6332023). The collected flowers were dried and stored in air tight container. The in vitro study was conducted on Center for Research on Molecular Biology and Applied Science, Thiruvananthapuram, Kerala.

Preparation of Veppam poo Ooral Kudineer (VOK):

4.2 g (1 varagan) of Flower of Azadirachta indica(Veppam poo) was taken and soaked in 90 ml(3 ounce) of hot water for 2 hours. Then it was filtered and the extract was used for analysis (12)

In Vitro Hepatoprotective Activity:

Cell culture:

HepG2 (Liver Hepatic cells) cell line was purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's Modified Eagles medium(DMEM) (Sigma Aldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

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Preparation of compound stock:
The extract solution VOK was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. Acetaminophen (20mM) was added to induce toxicity.

Cytotoxicity Evaluation:
After attaining sufficient growth, Acetaminophen (20mM) was added to induce toxicity and incubated for one hour, prepared extracts in 5% DMEM were five times serially diluted by two fold dilution (100µ1, 50µl, 25µl, 12.5µl, 6.25ul in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator (17).

Cytotoxicity Assay by Direct Microscopic observation:
Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method:
Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the sample content in wells were removed and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (18).

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x } 100}{\text{Mean OD of control group}}$$

Mean OD of control group

Statistical Analysis
All experiments were done in triplicates and results represented as Mean+/- SE. One-way ANOVA and Dunnets test were performed to analyse data.

RESULTS:

Cytotoxicity -MTT Assay
The hepatoprotective activity of VOK was assessed using Hepg2 cell lines. Hepatotoxicity was induced using 20mM Acetaminophen, which reduced the viability of the cells from 100% to 49.07%. The toxicity produced destroyed almost 51% of cells. The toxicity induced cells were then treated with VOK of varying concentration viz, 1.5, 3.1, 6.25, 12.5 and 25(µg/ml). On increasing dose concentration from 1.5 to 25 the cell viability increased from 51.38 to 84.52% in a dose dependent manner [Table – 1] [Figure - 1].

Microscopic observation
From the phase contrast images observed, the control shows healthy viable cells, the cells treated Acetaminophen with detected changes in the morphology like shrinking, granulation with necrosised cells. By treating the study drug VOK with increasing dose the cells shows regeneration. (Figure 2)

Table 1: Percentage Viability of VOK by MTT assay

Sample volume (µl/ml)	Average Absorbance @ 540nm	Percentage Viability
Control	0.7252	100
Acetaminophen	0.3559	49.07±0.42
VOK 6.25	0.3726	51.38±0.36
VOK 12.5	0.4217	58.15±0.20
VOK 25	0.4854	66.94±0.62
VOK 50	0.5657	78.01±0.22
VOK 100	0.6129	84.52±0.48

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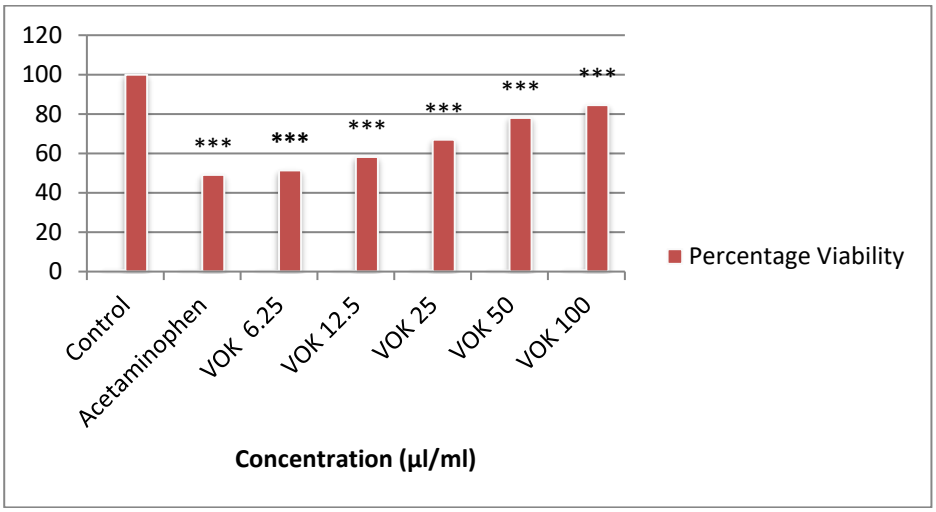

Figure 1: Graphical representation depicting the hepatoprotective effect of neem flower extract by MTT assay- Along Y axis Percentage viability, Along X axis varied concentration of neem flower extract.***p< 0.001 compared to Acetaminophen.

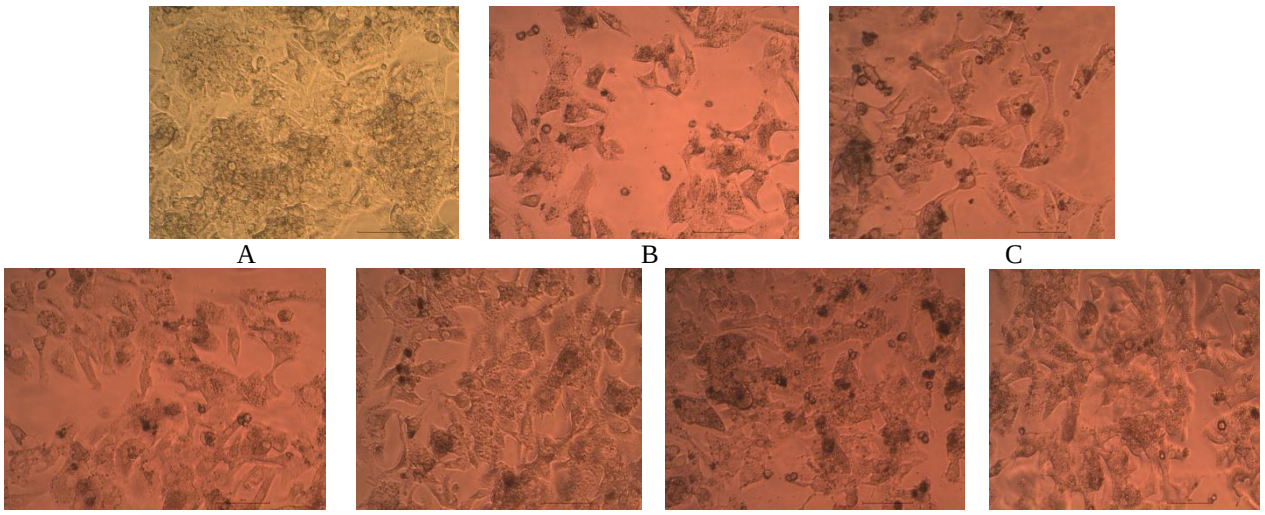

Fig.2 Photomicrograph of liver tissues: (A) Control (B) Acetaminophen induced cell (C) Acetaminophen + VOK (1.5 µl/ml) (D) Acetaminophen + VOK (3.1 µl/ml) (E) Acetaminophen + VOK (6.25 µl/ml) (F) Acetaminophen + VOK (12.5 µl/ml) (G) Acetaminophen + VOK (25µl/ml).

Discussion:

The liver is an organ responsible for metabolism, detoxification, and excretion, is pre-disposed to xenobiotics and, therefore, is a tissue susceptible to toxicity, which can cause morphological and functional changes (19, 20). APAP-induced HepG2 cells line is used as hepatotoxic model. It is widely known that APAP can induce liver injury through oxidative stress mechanism which is initiated by toxic metabolite N-acetyl-p-benzoquinone-imine (NAPQI) (21). The APAP cytotoxicity effect in the HepG2 cell line occurs through growth inhibition via caspase-mediated apoptosis and not necrosis, as observed in primary hepatocyte cell line (22). Herbal medicines are claimed to both treat and prevent diseases, which adds to a deep belief that these treatments are safe because they are "natural and gentle" and therefore, a harmless alternative to the conventional medicine (23). Due to their safety, ease of availability, affordability, and environmental friendliness, herbal medicines could be a viable treatment for the current liver issues. Many herbs are well-studied for their bioactive components and the mechanism of hepatoprotective activity.

Among them Azadirachta indica (Neem) is one of the most common medicinal plants that grow all over India. Neem has a role in the treatment of disorders like microbial infections, skin diseases, dental disorders, malaria, syphilis, leprosy and has antiseptic, hepatoprotective property (24, 25). Azadirachtin is the chemical ingredient which is found in all parts of the

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neem tree while nimbolide, β-sitosterol, chlorophylls and flavonoids such as rutin and quercetin are active constituents in the neem flowers(26-28) Dietary Neem flowers caused a marked increase in glutathione S-transferase activity in the liver(29) and also possess chemo preventive potential on mammary and liver carcinogenesis(30). As flower of neem was soaked in hot water for 2-12 hrs, Ooral kudineer is easy to prepare and to consume.

From this study, the extract VOK improved HepG2 cell viability in relation to the APAP group in a range of 51.38% to 84.52%. The extract VOK showed the best result, improving cell viability by about 84% in the APAP group. From previous studies, the quercetin and rutin, Nimbolide compounds of Azadirachta indica may be responsible for its hepatoprotective activity (31).Studies already proved that neem has antioxidant activity. Oxidative stress also plays a major role in APAP toxicity. Oxidative stress occurs when the generations of reactive oxygen species overwhelms the ability to detoxify the reactive intermediates or exceeds the capacity to repair the resulting damage (32).Hence it may be assumed that antioxidant activity (33) of neem extract may prevent hepatotoxicity cell injury induced by APAP exposure. As per literature, toxicity of APAP exposure due to excessive oxidative stress, as a result it may possible that antioxidant nature of veppam poo ooral kudineer (VOK) responsible for hepatoprotective action. Hence, the present study concludes that, veppam poo (Neem flower) ooral kudineer has a significant hepatoprotective activity.

Conclusion:

This present study concluded that, Veppam poo ooral kudineer effectively protects from toxic APAP-induced hepatotoxicity. This study was conducted as a preliminary study to evaluate the Siddha herbal formulation scientifically. Furthermore, in vivo and clinical studies are to be conducted to ensure the efficacy of VOK.

References

1. Kaur G, Alam MS, Jabbar Z, Javed K, Athar M (2006) Evaluation of antioxidant activity of Cassia siamea fowers. J Ethnopharmacol 108(3):340–348)
2. Yoon E, Babar A, Choudhary M, Kutner M, Pyrsopoulos N. Acetaminophen-Induced Hepatotoxicity: a Comprehensive Update. J Clin Transl Hepatol. 2016 Jun 28;4(2):131-42. doi: 10.14218/JCTH.2015.00052. Epub 2016 Jun 15. PMID: 27350943; PMCID: PMC4913076.
3. Herndon CM, Dankenbring DM. Patient perception and knowledge of acetaminophen in a large family medicine service. J Pain Palliat Care Pharmacother. 2014;28:109–116. doi: 10.3109/15360288.2014.908993.
4. Kaplowitz N. 2005. Idiosyncratic drug hepatotoxicity. Nature Reviews Drug Discovery 4: 489–499. DOI 10.1038/nrd1750
5. Bessems J.G. & Vermeulen N.P. 2001. Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. Crit. Rev. Toxicol. 31: 55–138. DOI 10.1080/20014091111677
6. Devarbhavi H. Drug-Induced Liver Injury Unique to India. Clin Liver Dis (Hoboken). 2021 Oct 15;18(3):108-110. doi: 10.1002/cld.1120. PMID: 34691395; PMCID: PMC8518348.
7. Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab Rev. 2012; 44:88–106. doi: 10.3109/03602532.2011.602688.
8. Pravin v. Gomase , vinod d. Rangari , p. R. Verma, phytochemical evaluation and hepatoprotective activity of fresh juice of young stem (tender) bark of azadirachta indica a. Juss, international journal of pharmacy and pharmaceutical sciences, vol 3, suppl 2, 2011
9. assem F, Aparna M. (2019). An update on therapeutic potential if neem and its active constituents. Era's j med res, 6: 369-410
10. hmad E, NidalJ. (2017). A review of chemical constituents and traditional usage of neem plant.Palest med pharm j, 2: 75-81.
11. Chattopadhyay RR, Sarkar SK, Ganguly S, Banerjee RN, Basu TK, Mukherjee A. Hepatoprotective activity of Azadirachta indica leaves on paracetamol induced hepatic damage in rats. Indian J Exp Biol. 1992 Aug;30(8):738-40. PMID: 1459654.
12. kannusamypillai ,pathartha Guna vilakam, first edition,2009, pg.no 675
13. Deva Aasirvadham Samuel, Marundhu Sei Iyalum Kalaiyum, Dept. of Indian Medicine and Homeopathy, Chennai. 2014, pg.no:91.
14. Thiagarajan R, Gunapadam Thathu- Jeeva vaguppu, Part II and III, Eighth edition, Dept. of Indian Medicine and Homeopathy, Chennai, 2013.
15. Thirunarayanan T, Introduction to Siddha Medicine, Centre for Traditional Medicine and Research, Chennai, 2012.
16. Sambasivam Pillai T.V, Dictionary of Medicine, Chemistry, Botany and Allied Sciences (Based on Indian Medical Science), Govt of Tamilnadu, Volume-1 Part 1, 1994, Page no: 1366.
17. Ansari, Shabnam; Hasan, Kazim1; Bhat, Sajad1. Anticancer, antioxidant, and hepatoprotective activity of Saussurea lappa, C.B. clarke (qust) on human hepatoma cell line. Journal of Cancer

Journal of Chemical Health Risks

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Research and Therapeutics 17(2):p 499-503, Apr–Jun 2021.

18. E.T. Arung, K. Shimizu, R. Kondo, Inhibitory effect of isoprenoid-substituted flavonoids isolated from Artocarpus heterophyllus on melanin biosynthesis,Planta Medica. 72 (9) (2006) 847–850.
19. Levy, B. Fisiologia, 6th ed.; Elsevier: Rio de Janeiro, Brazil, 2009.
20. Carvalho, H.F. Células: Uma Abordagem Multidisciplinar; Manole: Barueri, Brazil, 2005; p. 450.
21. Ramachandran A and Jaeschke H 2017 Mechanisms of acetaminophen hepatotoxicity and theirtranslation to the human pathophysiology J. Clin. Transl. Res. 3 157-69
22. Behrends, V.; Giskeodegard, G.F.; Bravo-Santano, N.; Letek, M.; Keun, H.C. Acetaminophen cytotoxicity in HepG2 cells is associated with a decoupling of glycolysis from the TCA cycle, loss of NADPH production, and suppression of anabolism. Arch.Toxicol. 2019, 93, 341–353. [CrossRef]
23. Izzo, A. A. , Hoon-Kim, S. , Radhakrishnan, R. , & Williamson, E. M. (2016). A critical approach to evaluating clinical efficacy, adverse events and drug interactions of herbal remedies. Phytotherapy research : PTR, 30(5), 691–700.
24. Murty KS, Rao DN, Rao DK, Murty LBG. A preliminary study on hypoglycemic and anti hyperglycemic effect of Azadirachta indica. Indian J Pharmacol 1978; 10: 247–250.
25. Pillai NR, Santha Kumari G. Hypoglycemic activity of Melia Azadirachta indica. Indian J Med Res 1981; 74: 931–933.
26. Srithanaudomchai, H., Kusamran, T., Kuakulkiat, W., Bunyapraphatsara, N., Hiransalee, A., Tepsuwan, A. and Kusamran, W.R. 2005. Quinone reductase inducers in Azadirachta indica A. Juss flowers, and their mechanism of action. Asian Pac. J. Cancer Prev. 6: 263-269
27. Subramanian, S.S, Nair, A.G.R. 1972. Melicitrin-A new myricetin glycoside from the flowers of Melia zadirachta. Indian J. Chem. 10: 452.
28. Sithisarn, P., & Gritsanapan, W. (2005). Free radical scavenging activity and total Flavonoid content of Siamese neem tree leaf aqueous extract from different locations. Mahidol University Journal of Pharmaceutical Sciences, 32, 31–35.
29. Kusamran WR, Ratanavila A, Tepsuwan A. Effects of Neem flowers, Thai and Chinese gourd fruits and sweet basil leaves on hepatic monooxygenases and glutathione S-transferase activities, and in vitro metabolic activation of chemical carcinogen in rats. Food Chem Toxicol 1998; 36: 475-484.
30. Tepsuwan A, Kupradinum P, Kusamran WR. Chemopreventive potential of Neem flowers on carcinogeninduced rat mammary and liver carcinogenesis. Asian Pac J Cancer Prev 2002; 3: 231-238
31. Chattopadhyay R R, Bandyopadhyay M. Possible mechanism of hepatoprotective activity of Azadirachta indica leaf extract against paracetamol-induced hepatic damage in rats: Part III. Indian J Pharmacol 2005;37:184-5
32. Cover C, Mansouri A, Knight TR, Bajt ML, Lemasters JJ, Pessayre D, Jaeschke H. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J Pharmacol Exp Ther. 2005;315:879–87. doi: 10.1124/jpet.105.088898.
33. Gayatri Nahak and R.K. Sahu, Evaluation of antioxidant activity of flower and seed oil of Azadirachta indica A. juss, Journal of Applied and Natural Science 3 (1): 78-81 (2011).