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African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 8 Number 21 8 June, 2014

ARTICLES

Research Articles

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Full Length Research Paper

Toxicological evaluation of extract of *Olax subsorpioidea* on albino Wistar rats

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This research work was undertaken in order to evaluate the toxicity effects of the leaf extract of *Olax subscorpioidea* on albino Wistar rats using the biochemical, haematological and histopathological indices. Five groups (A to E) of eight rats per group were used for this study. Animals in group A was administered with distilled water while the rats in groups B, C, D, and E were administered with 250, 500, 750 and 1000 mg/kg body weight of the extract of *O. subsorpioidea* via oral intubation for 28 days. Animals were subsequently anaesthetized in diethyl ether respectively and blood samples were collected for some biochemical and haematological assays, while the liver and kidney organs were isolated and processed for histopathological studies. Biochemical analysis revealed a significant decrease ($p<0.05$) in the levels of total bilirubin and alanine aminotransferase in the groups treated with 250 and 500 mg/kg body weight, while significant elevation ($p<0.05$) was observed in alkaline phosphatase, and albumin levels. Furthermore, haematological studies showed a significant reduction ($p<0.05$) in white blood cell count and haemoglobin level in the treated groups. Moreover, the group treated with 1000 mg/kg body weight of the extract exhibited a reduced ($p<0.05$) percentage mean cell haemoglobin and lymphocyte, while the percentage neutrophil was significantly increased ($p<0.05$). Histopathological studies conducted revealed that there was no significant damage on the liver and kidney tissues. The results suggest that extract of the leaf of *O. subscorpioidea* could alter the haematopoietic elements as well as some biochemical parameters and may not cause any adverse effect on the liver and kidney tissues.

Key words: *Olax subscorpioidea,* biochemical parameters, haematological parameters, histopathological studies.

INTRODUCTION

The use of medicinal plants in the treatment of various illnesses is due to their phytochemical constituents and can be traced back to antiquity (Yakubu et al., 2007). Due to the advancements in medicine, a lot of medicinal plants have been globally ignored. However, Africa and some parts in Asia utilize trado-medicine as an

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alternative for treatment. With the increasing discoveries of multifunctional herbs in these regions, herbal rebirth is thus taking over the world (Hoareu and DaSilva, 1999). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs (Doughari et al., 2008). *Olax subscorpioidea* belongs to the family of Olacaceae and can either be a shrub or tree; it is about 10 m in height and widely distributed in Nigeria, Zaire and Senegal (Ayandele and Adebiyi, 2010). It is known as “Mtungapwezi” in Swahili. It is called several names in Nigeria; for instance, it is called “Ukpakon” in Edo, “Ifon” in Southern Western Nigeria and “Aziza” in Nsukka (Ukwe et al., 2010). The plant is believed to possess medicinal properties; it is also reported to contain many bioactive compounds which are responsible for its diverse biological activities. Among them are tannins, alkaloids, saponin, flavonoids, glycosides, and steroids (Ayandele and Adebiyi, 2010). Like some other African plants, religious and superstitious believes are attached to it. The plant is often used as genital stimulants, pain killers, treatment of veneral diseases, rheumatoid arthritis, tooth aches, etc (Borokin and Omotayo, 2012). Investigation on the saline extract of the plant showed that it possesses membrane stabilizing property, whereas the sodium hydroxide extract possesses anti-protease activity (Oyedapo and Famurewa, 1995). Similarly, the antimicrobial activity of the ethanolic stem extract has also been reported (Ayandele and Adebiyi, 2007). Moreover, the plant root has been explored for the treatment of asthma and constipation (Okoli et al., 2007).

Since majority of the medicinal plants in Africa are believed to be multi-potent, they are used without knowledge of the potential toxic effects that may arise, thus, this study was aimed at evaluating the toxic effect of the ethanolic leaf extract of *O. subscorpioidea* by employing biochemical, haematological and histological indices in albino Wistar rats.

MATERIALS AND METHODS

Plant

The leaves of *O. subscorpioidea* were obtained from the campus of Covenant University, Canaan land, Ota, Ogun State, Nigeria in December 2012. The authentication was done by a botanist, Dr. A. C. Omonhinmin from the Department of Biological Sciences, Covenant University and a voucher specimen was also kept in the herbarium.

Reagent sources

Absolute ethanol used for the plant extraction was obtained from Sigma, Aldrich, USA, whereas all the kits used for biochemical assays were obtained from Randox Laboratories London, UK.

Plant extraction

The leaves were collected and air-dried for about three weeks and then smoothly homogenized using a domestic blender and was subsequently prepared for extraction. Five hundred grams (500 g) of the powdered leaves of *O. subscorpioidea* were extracted in 95% ethanol using a soxhlet apparatus. It was then concentrated at 50°C in a rotary evaporator to afford 95 g (19% yields) of the ethanolic extract (Ayandele and Adebiyi, 2010).

Experimental animals

Forty male Wistar rats, specific pathogen free, aged 2 to 8 weeks old were purchased from the University of Ibadan, Ibadan, Nigeria and were kept under standard environmental conditions (25 ± 21°C; 12/12 h light/dark cycle). Ten animals were kept in each cage and fed with standard diet (obtained from Graceline Feeds Ota, Ogun state) and clean water was given *ad libitum*. The animals were allowed to acclimatize for six weeks prior to the experiment. For experimentation, the animals were fasted overnight (Adebayo et al., 2010). The experimental animals were handled and used in accordance with the international guide for the care and use of laboratory animals (National Institute of Health, 1985).

Experimental design

The animals were divided into 5 groups (A to E) of 8 rats per group. Group A was the control group whose animals were treated with distilled water, while animal subjects in groups B, C, D and E (Test group) were administered 250, 500, 750 and 1000 mg/kg body weight of ethanolic leaf extract of *O. subscorpioidea* (dissolved in distilled water), respectively, all groups were fed and treated for 28 days.

Blood sample and organ collection

Animals were sedated with diethylether, blood samples were collected via cardiac puncture into two sets of lithium heparinized bottles. Plasma was obtained in one set by centrifuging the blood at 10,000 revolutions/min for 15 min and stored at -20°C in Eppendorff bottles until required for biochemical assays, while the whole blood was used for haematological studies. The organs (liver and kidney) were collected and placed in 10% formalin (Aliyu et al., 2007; Charity et al., 2012).

Biochemical assay

An auto-analyzer (Archem BM240, Turkey) was used to assay for biochemical parameters which include: aspartate amino transferase (AST) (Bergmeyer et al., 1986A), alanine aminotransferase (ALT) (Bergmeyer et al., 1986b), alkaline phosphatase (ALP) (Tietz et al., 1983), total bilirubin (Doumas et al., 1973), albumin (Doumas et al., 1971), and urea (Krieg et al., 1986).

Haematological assays

An automated haematology system analyzer (Archem BM240, Turkey) was used to assay for white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean cell volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT), mean cell haemoglobin (MCH), percentage lymphocyte (% LYM), neutrophil (NEU) (Baker et al., 1998).

Histopathological studies

The livers and kidneys tissues were fixed in normal saline for 72 h

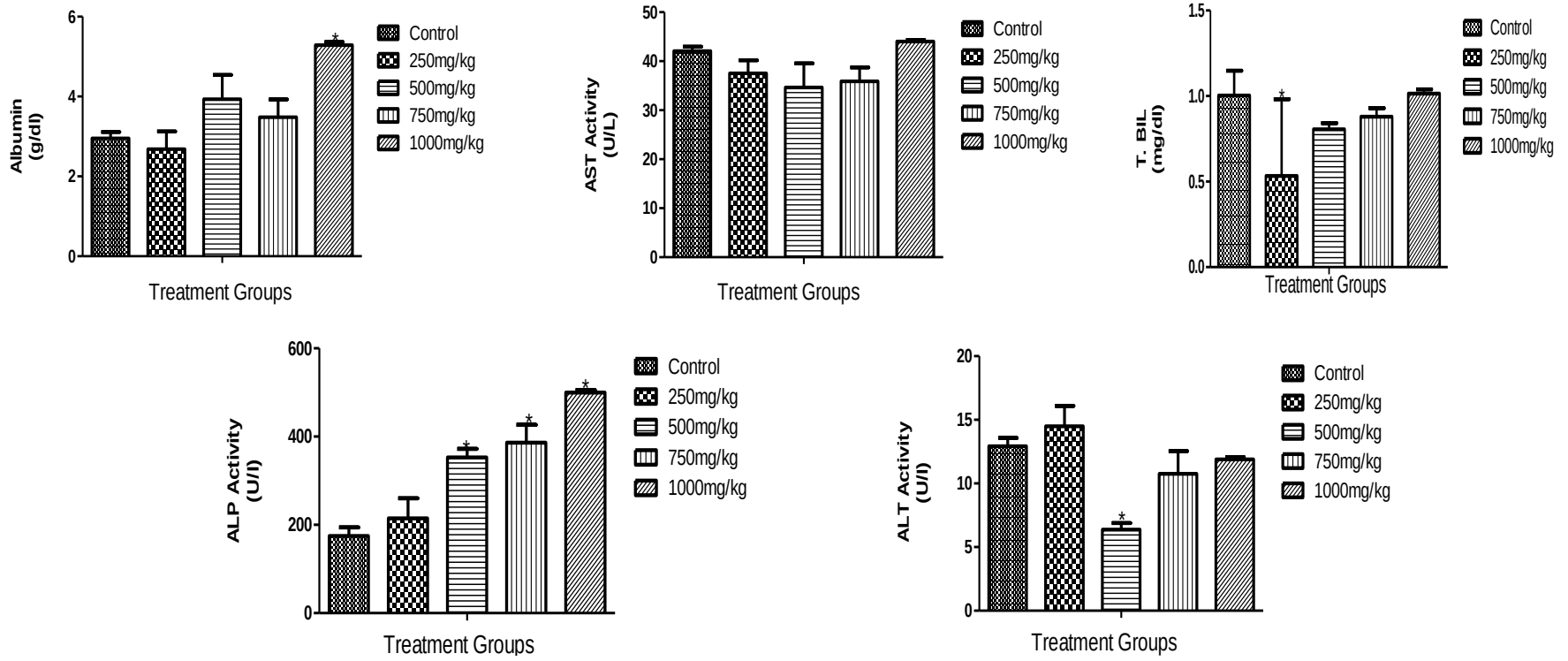

Figure 1. Effect of leaf extract of *Olax subscorpioidea* on liver functions of albino Wistar rats.
AST, aspartate aminotransferase; T.BIL, total bilirubin; ALP, alkaline phosphatase; ALT, alanine amino transferase. Values represent mean ± SEM of 8 replicates; *p < 0.05.

and sliced into a thickness of 2.1 mm. The tissues were dehydrated with alcohol of graded concentrations. They were further treated with paraffin wax and cast into blocks; sections of the tissues were then cut on a microtome to 5 µm. these were later attached to a slide and allowed to dry. The sample slides were subsequently stained with haematoxylin-eosin and examined under a light microscope; photomicrographs of the samples were recorded (Aliyu et al., 2007; Charity et al., 2012).

Statistical analyses

All data were expressed as mean ± standard error of mean (SEM), a probability of $p < 0.05$ being considered significant. Group comparisons were done using the analysis of variance (ANOVA) test. Tukey's post hoc test was carried out to analyze significance of difference between different groups.

RESULTS

Biochemical analysis

The effects of the extract on liver functions in blood of rats were assessed (Figure 1). ALT, AST and total bilirubin were not significantly elevated ($p > 0.05$) when compared with the control group although there was a significant reduction ($p < 0.05$) in ALT and total bilirubin in the group treated with 500 and 250 mg/kg body weight of extract, respectively. A significant increase ($p < 0.05$) in ALP was observed in the groups treated with 500, 750 and 1000 mg/kg body weight of extract, while the level of albumin in the group treated with 1000 mg/kg body weight of extract was also significantly increased ($p < 0.05$). Analysis of the urea concentration revealed that there was no significant change observed in blood urea nitrogen (Figure 2).

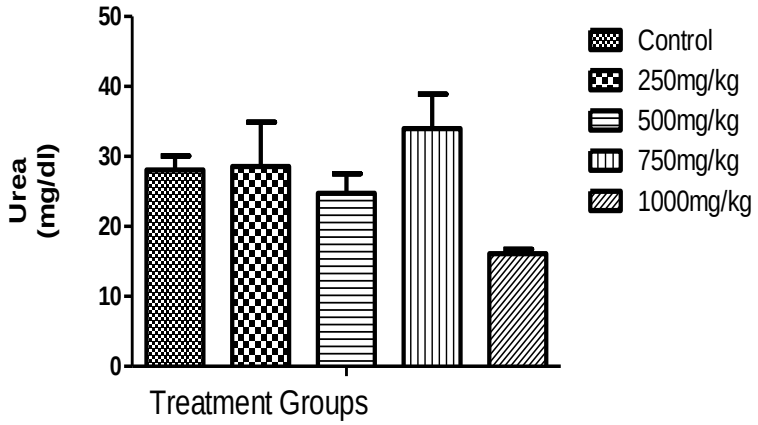

Figure 2. Effect of leaf extract of *Olax subscorpioidea* on urea level of albino Wistar rats. Values represent mean ± SEM of 8 replicates; $p > 0.05$.

(Figure 2).

Haematological analysis

Haematological parameters such as: red blood cell count (RBC), mean cell volume (MCV), platelet count (PLT) and mean corpuscular haemoglobin concentration (MCHC) were not significantly affected ($p > 0.05$), but there was significant decrease ($p < 0.05$) in WBC count of all treated groups. Similarly, there was a significant reduction ($p < 0.05$) in haemoglobin level in groups treated with 250, 500 and 1000 mg/kg body weight of extract. Moreover, MCH and percentage lymphocyte significantly dropped ($p < 0.05$) in the group treated with 1000 mg/kg body weight of extract, while percentage neutrophil was significantly elevated ($p < 0.05$) in the group treated with 1000 mg/kg body weight of extract (Figure 3).

Histopathological studies

No significant damage was observed in rats' liver and kidney tissues in groups treated with ethanolic leaf extract of *O. subscorpioidea* when compared with the control groups (Figures 4 and 5).

DISCUSSION

Toxicological assessment of potential drugs, herbs and extracts are necessary to establishing the safety limit of these substances in animals. They are commonly used to assess the possible health risk in humans, caused by intrinsic adverse effects of compounds or plant extracts (Klassen and Eaton, 1991). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are specific markers of hepatic injury and hepatocellular necrosis (Blackwood, 2001). The insignificant elevation in the activities of ALT and AST reveals that the ethanolic extract may not be harmful to the liver, because increase in plasma ALT and AST activity are markers for hepatic damage (Rej, 1989), though the cause of the reduction of ALT activity in the group treated with 750 mg/kg body weight of the extract is unknown. This result however agrees with the study conducted on leaf of *Chrysophyllum albidum G* which also decreased ALT level in Wistar rats (Adebayo et al., 2010). The decrease observed may be attributed to the ability of the extract to alter the hepatocytes and down regulate its production which could be beneficial. ALT is localized in the cytosol of the hepatocytes and more sensitive marker of hepatocellular damage as opposed to AST from the mitochondrial which can also be produced from other tissues like heart, kidney and pancreas other than the liver (Mabeku et al., 2007). The contrasting dose dependent significant increase of alkaline phosphatase activity in the group that received the higher doses as shown in Figure 1 was also observed by Adedapo et al. (2009). ALP is a metalloenzyme whose activity change in the blood is xenobiotic-sensitive, because it is a glycosylphosphatidylinositol (GPI) anchored protein of the microsomal and cellular membrane of hepatocyte, this makes it susceptible to cleavage by phospholipase D, proteases and bile acids (Rej and Bretaudiere, 1980). Flavonoids present in the leaf extract of *O. subscorpioidea* have been implicated to induce the activity of ALP. Since ALP is also synthesized by other tissues of the body, a dose related rise in ALP activity may not be due to liver damage (Ayandele and Adebiyi, 2007; Hsu et al., 2009). This result is supported from the histological assessment of the liver tissue where no obstructive jaundice and intrahepatic cholestasis as a consequence of high level of ALP was observed. The high concentration of albumin observed in group treated with 1000 mg/kg body weight of extract poses the risk of high oncotic blood pressure at higher doses of the plant

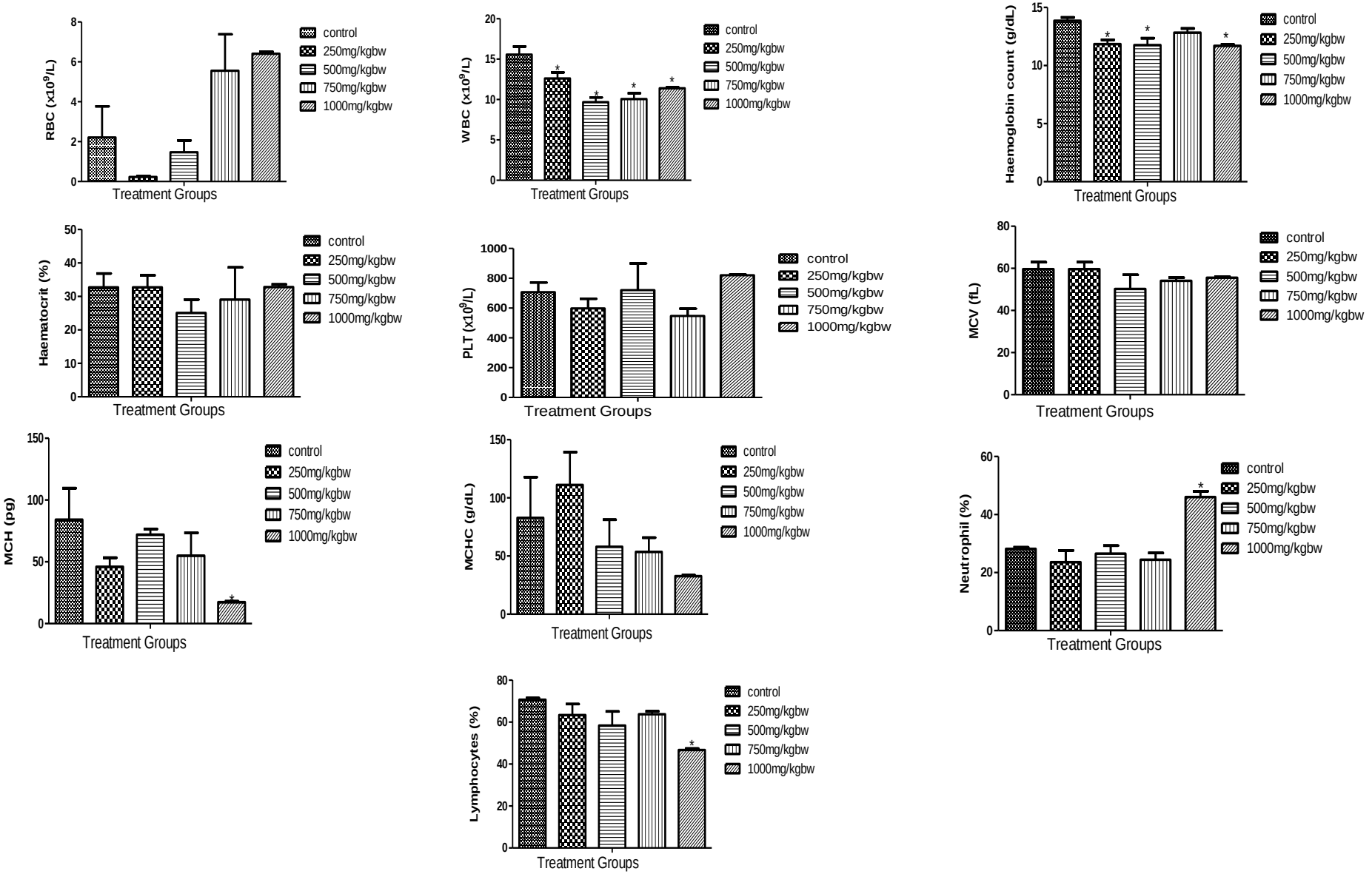

Figure 3. Effect of the leaf extract *O. subscorpioidea* on the haematological parameters of albino Wistar rat. Values represent mean ± standard error of mean (SEM) of 8 replicates; *p < 0.05. RBC, red blood cell count; WBC, white blood cell count; PLT, platelet count; MCV, mean platelet volume mean corpuscular hemoglobin, MCH; MCHC, mean corpuscular hemoglobin concentration; LYM%, percentage lymphocyte.

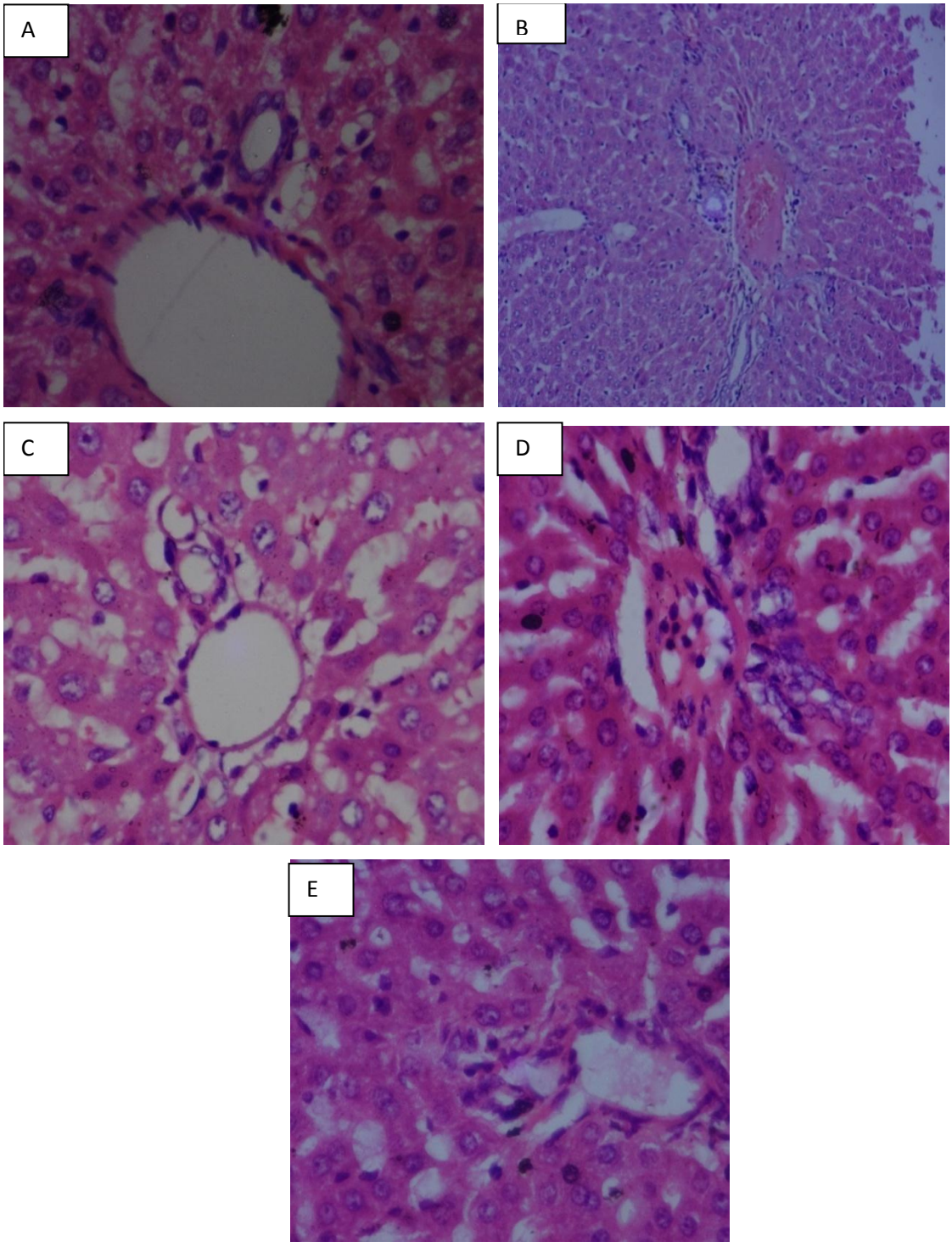

Figure 4. Photomicrograph of the liver tissues (H&E x160) of albino Wistar rats treated with extract of *O. subscorpioidea.* Figures A to E show the tissues of rats treated with 0, 250, 500, 750 and 1000 mg/kg body weight respectively).

plant extract. Albumin apart from being a useful indicator of the integrity of glomerular membrane is also important in determining the severity of the disease (Adedapo et al., 2005). Increased albumin may be primarily due to high synthetic ability of the liver. The elevated level observed in this group suggests an enhancement in the synthetic function of the liver (Kaplan et al., 1988). The non significant increase in urea during the treatment duration may be

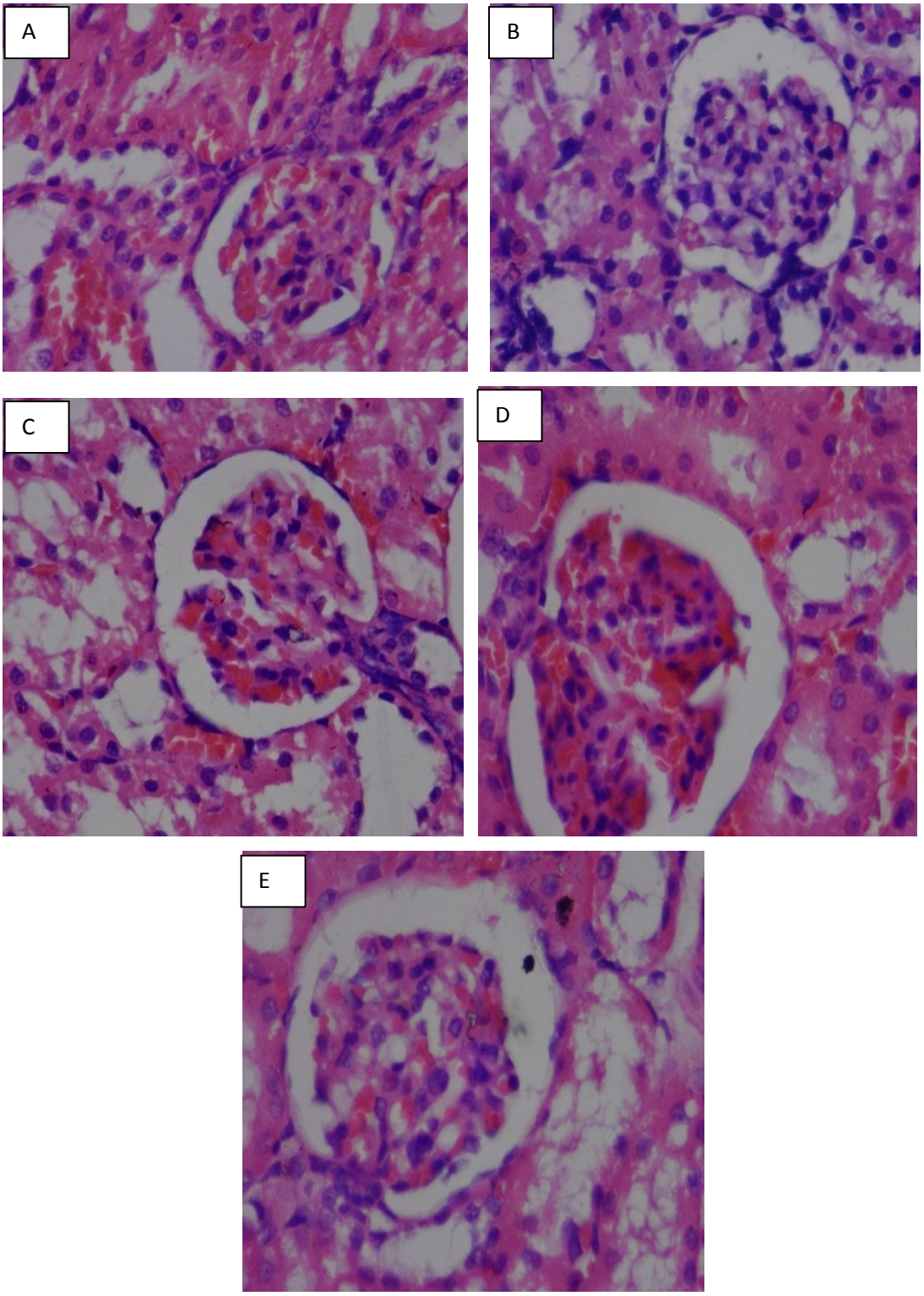

Figure 5. Photomicrograph of the kidney tissues (H&E x160) of albino Wistar rats treated with extract of *O. subscorpioidea.* Figures A to E show the tissues of rats treated with 0, 250, 500, 750 and 1000 mg/kg body weight respectively).

may be attributed to the impairment of the urea cycle leading to the reduced production of urea suggesting that the extract may not possess any phyto-constituent obstructing the urea cycle (Adebayo et al., 2003). The result corroborates the histopathological findings of the kidney tissues where no significant damage was observed.

The decrease in WBC count and lymphocyte makes the animal vulnerable to infections caused by pathogens (Anofi and Olugbenga, 2011). The decrease in the values of WBC in the treated rats indicates that the rats were fighting against some forms of infection probably induced as a result of administration of this plant extract. In a similar study conducted by Iweala and Obidoa (2009), they observed that the presence of phytsterols and flavonoids in the leaf extract of *Gompholobium latifolium* might possibly interfere with the process of WBC synthesis. The presence of these phytochemicals may however play synergistic roles in mediating this activity. The decrease in free haemoglobin level of groups B, C and E and MCH in group E reveals that the plant extracts may contain some harmful phytochemicals, posing the risk of anaemia although the insignificant change observed in the quantity of red blood cell is paradoxical. Saponins have been implicated to be highly toxic when injected into the blood stream and cause haemolysis of the red blood cells and eventually destroying the cells (Itodo et al., 2011). This therefore suggests that saponins from the plant extract might be responsible for the significant reduction of the haemoglobin count. Percentage neutrophil upsurge as seen in group E is a signal for immune-attack and anaemia (Harshida et al., 2013). Thus, further increase in the dose of the extract will compromise the immune system and the free blood haemoglobin status.

In conclusion, this study has demonstrated that the ethanolic leaf extract of *O. subscorpioidea* may not cause deleterious effects on the liver and kidney tissues, but could alter the haematopoietic elements as well as some biochemical parameters. Further investigation is needed to establish the bioactive compounds of the extract involved in mediating these biological effects.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Full Length Research Paper

Pharmaceutical industry capacity utilization in Nigeria

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The performance of any enterprise is influenced by the environment in which it is located. The last two decades has witnessed the pre and post democratic governance in Nigeria as well as a number of government initiatives aimed at addressing some challenges facing the health delivery system of the country. The purpose of this paper was to study the capacity utilization of the pharmaceutical industry within the period to understand whether there was or no association in the performance with these changes. A survey of the capacity utilization in the production of solid and liquid dosage forms was carried. A study of the availability and distribution of scientific workforce in the industry was also undertaken. Interviews were held with some individuals to gain more insight in the activities of the pharmaceutical industry. The scientific workforce within and among organizations varied considerably and the variation was influenced by perceived benefits and opportunities. The study showed that the capacity utilization of the pharmaceutical industry increased steadily but marginally over the period. There was reason to believe that the marginal increments were influence by the government policies and initiatives during this period, but could be better if some economic investments such as steady electricity were made. It was concluded that a sustained support and provision of infrastructures by the government together with trained and motivated scientific workforce are important in improving the capacity utilization in the pharmaceutical industry.

Key words: Government initiatives, infrastructures, scientific workforce, capacity utilization, pharmaceutical industry.

INTRODUCTION

Nigeria is a relatively large country with an estimated population of 169 million and it is endowed with natural resources, high levels of human and social capital. However, it is plagued with a very high incidence of disease, poverty and malnutrition and has lower life expectancy than some other African countries of comparable economy (WHO, 2013).

The role of the pharmaceutical industry in a country such as Nigeria in the provision of safe, pure, quality and efficacious products to meet the healthcare need of the populace cannot be over-emphasized. Provision of essential medicines by this sector would curb infiltration of the market with spurious and substandard products and would also enhance the economy.

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In realization of key role the availability of essential drug plays in health system, the government of Nigeria has made more efforts in empowering the pharmaceutical industry in the last two decades than was ever made in the history of the country. Specifically, the Petroleum Trust Fund (PTF) was established by the Federal Government in 1990s to provide infrastructural incentives to all sectors of the economy, as a palliative to the increased pump price of petroleum and the pharmaceutical industry was a key beneficiary (LFN, 1994). The year 1990 witnessed the adoption and launch of the maiden National Drug Policy (NDP) for Nigeria to address the inadequacies in drug availability, supply and distribution. The establishment of National Agency for Food and Drugs Administration and Control (NAFDAC), the publication of Essential Drug List (EDL) and a National Drug Formulary (NDF) resulted from the policy. In the first revised National Drug Policy (NDP, 2005), it was anticipated that by 2008, the local pharmaceutical industry would have realized a production capacity of 70% to satisfy at least 60% of national drug requirements of essential drugs, while the balance was to be exported (NDP, 2005). Consequently, a number of essential drugs that the local manufacturing industry has the capacity to produce have been put on import prohibition list to encourage the local manufacture and improve on the capacity utilization of sector (NCS, 2014).

A number of articles are available in the literatures addressing the effect of the various initiatives of the government on the manufacturing sector (Akinlo, 1996; Essien, 2005; Inegbenebor, 1995; Kayode, 1987), but none regarding the pharmaceutical subsector is currently available. Additionally, a typical pharmaceutical industry is home to interdisciplinary professional workforce, whose qualifications, motivation and competencies have impact on the quality of products and services rendered by the pharmaceutical industry. The present study was an investigation on the profile of the scientific workforce and the effects of some government initiatives in the last two decades on the capacity utilization of the pharmaceutical industry.

MATERIALS AND METHODS

The study instruments used were questionnaires and interviews, which contained both closed and open ended questions.

Capacity utilization study

Seventy (70) copies of questionnaires were administered to representatives of the pharmaceutical manufacturing companies. The survey covered public liability companies (PLC), limited liability companies (LTD) and non-for profit or non-governmental (NGO), which may be a multinational, international or indigenous company. Convenience method of sampling was employed to optimize the results of the survey. The questionnaire was filled by officers who had the relevant information on the following areas: the nature of pharmaceutical business, age of the organization, line of products, annual installed and used equipment capacities, staffing and their productivity, number of shifts per day, sources of raw and packaging materials, research and development activities by Nigerian pharmaceutical industry and the use or otherwise of locally fabricated machinery.

Survey on workforce

Two hundred and fifty (250) copies of the questionnaires were administered to individual employees of the pharmaceutical industry. Self-report questionnaires method of Michigan Organization Assessment Package was adapted for the individual questionnaires (Michigan, 1975). The questionnaire sought information from individual employees working in the pharmaceutical industry in the following areas: age and sex, work experience, job related challenges, meaningfulness of the job, responsibility, variety and skill task identity and feedback, autonomy, work influence, pace control, role conflict and clarity, task uncertainty and interdependence, resource and skill adequacy and competencies. Closed questions were predominantly used, as appropriate, for greater precision and ease of analysis. Various persons including pharmacists, chemists and microbiologists working in sampled pharmaceutical industry received the questionnaires. Engineers and other scientists not directly involved in manufacturing and distribution of the pharmaceutical products were excluded from the study. Pre-tested questionnaires were modified to keep the questionnaires short and simple.

Interviews

Interviews were also held with selected individuals who have spent over fifteen years in the industry in strategic positions and understood the dynamics of pharmaceutical business in Nigeria.

RESULTS

Twenty eight organizations comprising 9 multinational/ international and nineteen (19) indigenous companies responded to the questionnaire. Fourteen (50.0%) and 24 (85.7%) of the organizations manufacture tablets and liquid preparations, respectively. Capsules and sterile products, respectively were manufactured in 4 and 3 of the facilities surveyed. Twenty two organizations use own facilities for the manufacture of their products while the remaining ones utilized third party facilities.

Installed capacity and capacity utilization

Tables 1 and 2 show the one factor analysis of variance (ANOVA) of installed capacity utilization of machinery in liquid and tablet production in the sector over the last twenty years. The capacity utilization in the production of liquid preparations was between 33.08±0.9 to 55.6±1.14%, while the corresponding value for the tablets was 35.71±1.8 to 49.93±2.4%. The F (19, 460) was 51.14 at P-value of 0.00 compared to the F critical of 1.61 for the liquid production line, while in the tablet production line, the F (19, 260) value was 4.37 compared to F critical of 1.63. Figure 1 shows the capacity utilization in the

Table 1. ANOVA of capacity utilization in the production of liquids single factor ANOVA of capacity utilization liquid production line in Nigeria Pharmaceutical Industry from 1993-2012.

Summary (Group)	Count	Sum	Average	Variance
1993	24	857	35.70833	19.08514
1994	24	837	34.875	22.9837
1995	24	794	33.08333	19.9058
1996	24	827	34.45833	26.51993
1997	24	857	35.70833	19.08514
1998	24	857	35.70833	19.08514
1999	24	928	38.66667	32.49275
2000	24	997	41.54167	50.1721
2001	24	1082	45.08333	65.38406
2002	24	1204	50.16667	49.88406
2003	24	1285	53.54167	64.25906
2004	24	1318	54.91667	38.42754
2005	24	1335	55.625	31.54891
2006	24	1330	55.41667	43.73188
2007	24	1288	53.66667	28.84058
2008	24	1297	54.04167	25.60688
2009	24	1295	53.95833	39.51993
2010	24	1290	53.75	48.80435
2011	24	1252	52.16667	44.05797
2012	24	1191	49.625	26.7663

ANOVA (Source of variation)	SS	df	MS	F	P-value	F crit
Between Groups	34775.79	19	1830.305	51.11432	1.9E-100	1.609196
Within Groups	16471.71	460	35.80806	-	-	-
Total	51247.5	479	-	-	-	-

production of tablets and liquid products. The average capacity utilization decreased in 1995 and 1996. In liquid manufacture, the capacity utilization witnessed marginal increase from 1997 to 2012, and then declined of the capacity utilization in tablets decreased in 2002. Figure 2 shows the combined average capacity utilization of the tablets and liquid preparations. The Figure showed a generally slow but steady rise in the capacity utilization of the period, declining only in 2012.

Infrastructural facilities

Source of energies

Pharmaceutical manufacturers in Nigeria use public and own energy sources. Three (12%) of the respondents use the electricity supply from the Power Holding Company of Nigeria (PHCN), formerly known as National Electric Power Authority (NEPA), while an organization (4%) used own generating plant. Twenty four respondents (96%) depended on PHCN and own plant for energy supply. PHCN electricity supply met 4 to 64% of the annual energy requirements of the respondent organizations and was considered cheaper than own source of energy.

Water supply

Ninety percent of the respondents have their own water source and do not rely on supply from the public main.

Access road

The pharmaceutical companies have access roads that are not maintained in most of the cases.

Information and telecommunication technology (ICT)

A 70% of the respondents were not satisfied with telecommunication services when Nigeria Telecommunication Limited (NITEL) was the sole provider of telecommunication services in the 1990s. The respondents claimed that the global system of communication (gsm) has made information and telecommunication technology more accessible. All the 28 companies that responded have at least desktop and or personal computers as well as other electronic devices for ICT management in the organizations.

Table 2. ANOVA of capacity utilization in the production of tablets single factor ANOVA of capacity utilization tablet production line in Nigeria Pharmaceutical Industry from 1993-2012.

Summary (Group)	Count	Sum	Average	Variance
1993	14	520	37.14286	9.362637
1994	14	536	38.28571	18.98901
1995	14	500	35.71429	47.45055
1996	14	502	35.85714	64.13187
1997	14	688	49.14286	189.3626
1998	14	684	48.85714	119.0549
1999	14	677	48.35714	36.24725
2000	14	655	46.78571	37.1044
2001	14	648	46.28571	48.68132
2002	14	624	44.57143	27.64835
2003	14	631	45.07143	30.07143
2004	14	635	45.35714	51.78571
2005	14	645	46.07143	50.68681
2006	14	659	47.07143	93.91758
2007	14	674	48.14286	89.82418
2008	14	679	48.5	116.1154
2009	14	699	49.92857	80.07143
2010	14	688	49.14286	109.3626
2011	14	694	49.57143	112.5714
2012	14	620	44.28571	64.83516

ANOVA (Source of variation)	SS	df	MS	F	P-value	F crit
Between groups	5805.414	19	305.5481	4.373487	1.69E-08	1.626697
Within groups	18164.57	260	69.86374	-	-	-
Total	23969.99	279	-	-	-	-

Some specific interventions

The establishment of the National Agency for Food and Drug Administration and Control (NAFDAC) in the early 1990s was the most significant decision of the government against the proliferation of fake and substandard pharmaceuticals in the country. All the respondents acknowledged the positive impact of the agency on drug business in Nigeria as it sanitized the drug manufacturing and advertisement to a great extent.

Petroleum (special) trust fund

Opinion was divided on the impact of the Petroleum Trust Fund (PTF) on the manufacturing sector. Seventy eight percent (78%) of the respondent organizations had marginal improvement in the capacity utilization of their facilities that was traced to the intervention.

Other interventions

Personal interview with some key players in the industry revealed that the pharmaceutical industry believes in the ability of the intervention fund to the industry by the federal government in the form of NAFDAC-Central Bank of Nigeria (NAFDAC-CBN) fund to turn things around for better. It was generally agreed that the import prohibition of some essential drug impact positively on the industry. The President's Emergency Plans for AIDS Relief (PEPFAR) funds and the current World Health Certification drive by some pharmaceutical companies are areas capable of significantly improving the capacity utilization in the pharmaceutical industry subsector of the economy.

Research and development profile of the pharmaceutical industry in Nigeria

Twenty respondent organizations (71.43%) carry out marketing related research with 1 to 5% of their profit after tax annually. Research on drug discovery was not carried out in any of the respondent organizations, but limited formulation studies happened occasionally when an excipient is substituted. Ten (35.7%) of the respondents use locally fabricated equipment such as

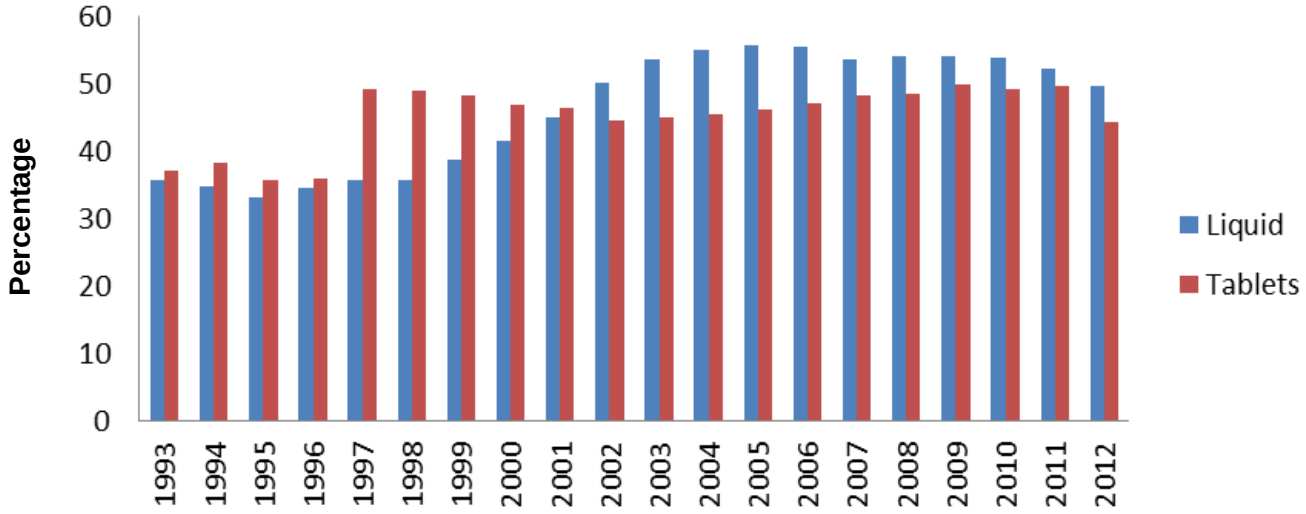

Figure 1. Average capacity utilization for tablets and liquid preparations from 1993-2012.

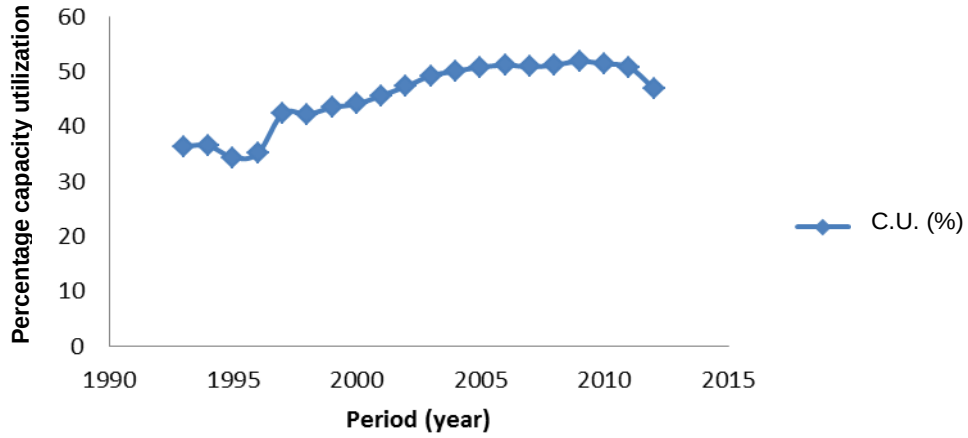

Figure 2. Combined average capacity utilization for tablets and liquid preparation.

mixing tanks, mixers, scoops, stainless steel containers and simple liquid filling equipment for their production. The survey showed that over 60% of the equipment used in the manufacture of liquid preparations was imported, while tablets and capsules are manufactured using imported equipment.

Scientific workforce in the pharmaceutical industry

One hundred and forty four (144) of the 250 questionnaires were returned from the scientists working in the pharmaceutical manufacturing establishments. Thirty percent of the respondents were 23 to 33 years old, while the remaining respondents were older but less than 55 years. Two of the respondents had PhD, one had Fellowship of the Postgraduate College of Pharmacists and 103 have other postgraduate qualifications. The survey revealed that 28 (19.4%), 17 (11.81%), 72 (50.0%) and 27 (18.75%) of the respondents had worked in the industry for 1 to 4, 5 to 10, 11 to 20 and over 20 years, respectively. The workforce distribution of the respondents was 11, 39 and 50% in production, quality control and marketing comprising pharmacists (51%), chemists and biochemists (39%), microbiologists (8%) and other scientists (2%). Job satisfaction among the scientific workforce ranged from 40 to 100%. The study showed that the employee in marketing were more satisfied with their jobs than their counterparts in either production or quality control.

Job security

Over 80% of the respondents felt secured in their current jobs and do not anticipate being fired or laid off by their

organizations, while 20% were not sure. About 75% of the workforce in marketing was likely to look for a higher pay opportunity in the next one year. The respondents claimed to have achieved 75 to 100% of the annual target rated on options of less than 50, 50, 75, and 100% or above 100% achievement of annual target. Production and quality control workers met their targets more than their counterpart in marketing and sales.

Mental acuity utilization

The survey showed that mental acuity utilization in the area of competencies was less than 50%. Managerial capabilities of the respondents were rated as adequate by 33.3% (48), while the remaining 67.7% (96) claimed that management skills were inadequate for their current responsibilities. The respondents were evenly split in the rating of the on-the-job training as either adequate or inadequate.

Determinants of career choice

The determinants of choice of area of practice within the industry were monetary reward (40%), opportunity for promotion (18%), job security (16%), public perception (15%), job contents and challenges (11%).

Generally, benefits such as incentives bonuses, company automobile, stock options and potentials for and advancement more often than not account for how opportunities within the sections of the industry are rated and are the driving forces for seeking employment opportunities by scientists.

DISCUSSION

Both indigenous and multinational establishments are involved in the manufacture of pharmaceuticals in Nigeria and over 90% of them are concentrated in Lagos, the commercial nerve of the country, and the contiguous states. The industry engages in the secondary manufacture of solid and liquid dosage forms. There are more manufacturers of liquid preparations than of solid probably, because of the few unit operations involved in liquid manufacture and hence relatively lower manufacturing cost. Liquid preparation equipment have more flexibility in usage to make different products than either tablet or capsule and this might explain why the capacity utilization of liquid producing equipment was relatively higher than those of the tablets. While the same mixing tank and a filling and capping line can be used for a number of products, the use of a tableting machine for tablets of different sizes and shapes will require change parts. The results showed that there was a general marginal increase in the capacity utilization over the period. This was probably due to several measures by the government aimed at improving the productivity of the industry. The year 1995 was a very challenging time in Nigeria due to political instability and power struggle, which impacted on many sectors of the economy; and this might explain why the capacity utilization declined in 1995 (Anon, 1998; Olowe, 1995). It was also probable that the new requirements for manufacture and advertisements of drugs, introduced by NAFDAC, were yet to be understood and complied with by the manufacturers.

Manufacturing productivity growth and capacity utilization are two major phenomena that are intertwined such that the higher the capacity utilized, the larger the outputs that are produced and the faster the growth of manufacturing productivity (Bamikole, 2012).

Generally, capacity utilization of 70 to 75% is required for a sector to experience productivity growth (Bamikole, 2012). Manufacturing productivity growth is the increase in the efficiency and productive capacity of the manufacturing sector.

Slackening domestic demand and lack of foreign exchange to import raw materials or imported inputs can negatively affect capacity utilization (Essien, 1990) and this seems to be the case with the pharmaceutical industry. The exchange rate and foreign reserves may influence manufacturing productivity growth and capacity utilization, if the naira depreciates against the dollar, imports become exorbitant and production at home may be halted. This may be the case with the low capacity utilization in the pharmaceutical industry in spite of the fact that some products have been placed under import prohibition list, to encourage their local manufacture. Investment in infrastructure has a positive impact on manufacturing productivity, while inefficient investment in economic infrastructure (electricity and transport) has a negative effect on manufacturing productivity (Adenikinju and Olofin, 2000; Ukoha, 2000; Mojekwu and Iwuji, 2012). This indicates that government interventions must be all inclusive to realize high manufacturing activity in the pharmaceutical subsector. The current wave of huge investments by some local manufacturers to upgrade their facilities to internationally accepted standard is a good development and government can do well to patronize them and also encourage donor agencies involved in procurement of pharmaceuticals to turn to local manufacturers of such articles. In this regard, establishment of good channels of communication between the government and the stake holders in the industry would be very crucial. It has been reported that the current local manufacture of essential drugs meets only 30% of the country's essential drug needs (Thepahrmaletter.com) which means that reasons other than lack of patronage by the government are responsible for the sector not meeting her target of producing 60% of the country's need of essential drug (PMG-MAN). Interestingly, two third of the over 34 million world population of people living with HIV/AIDS are in the

Sub-Saharan Africa (www.theglobalfund.org). Moreover, there is substantial allocation of funds for malaria and tuberculosis that the local industry can provide marching drug component requirements and thereby increase capacity. There would be sense in developing requisite expertise that would effectively manage the scarce resources at the disposal of pharmaceutical industry and for the government to take a holistic approach in re-vamping the ailing industry. Optimum local production of the essential medicines will not only enhance access to good quality medicines, but will also create employment opportunities. Worthy of mention is the need for collaboration between the industry and the academia in the area of research and development so as to take advantage of the locally available abundant resources that could serve as excipients and packaging materials.

Scientific workforce in the pharmaceutical industry

Company size and product lines are different and there exists considerable variability in the utilization of pharmacists and other scientists within industry. Disparity in benefits within and among organizations can be a challenge to workforce availability, which can impart negatively on the quality of goods and services delivered by the industry. The manufacture of safe and efficacious medicines cannot take place without the input of skilled and motivated scientific workforce. One of the critical aspects of the long term sustainability of the pharmaceutical industry is enlargement of human capitals that can satisfy the diverse undertakings in the pharmaceutical manufacturing system. There must also be a continuous linkage between the industry and the academia to be able to focus on training manpower that will meet the manufacturing and research skill needed in the industry.

Conclusion

A study of the capacity utilization in the last two decades in the pharmaceutical industry in Nigeria was carried out. There was association between the performance of the pharmaceutical industry in Nigeria and interventions of the government over the last two decades, but the influence was marginal on the capacity utilization of the pharmaceutical industry over the period. Full benefit of any intervention by the government can be realized if there is corresponding economic investment in infrastructures particularly electricity and the stabilization of naira. A focused search for and development of excipients from local sources can boost capacity utilization of the pharmaceutical industry.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Full Length Research Paper

Anti-oxidant and cytotoxic activity of *Cassia nodosa* Buch.-Ham. ex Roxb. and some of its pure constituents

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Cassia nodosa Buch.-Ham. ex Roxb. is an ornamental plant which belongs to the family Fabaceae. It does not have the appropriate phytochemical and biological investigation. This was the first time to investigate anti-oxidant and cytotoxic activity of _Cassia nodosa_ flowers, leaves, stem bark methanolic extracts and their fractions (petroleum ether, methylene chloride, ethyl acetate and n-butanol). Besides, some pure constituents isolated from _C. nodosa_ were assessed as anti-oxidant and cytotoxic agents. Remarkable results were obtained specially for stem bark methanolic extract as a strong cytotoxic agent against MCF-7 and VERO cell lines. Chrysophanol (IV) displayed the highest activity as anti-oxidant (anti-hemolytic and DNA protective agent). Anti-oxidant activity of it was higher than ascorbic acid which was the positive control. This was the first time to isolate Kaempferol-3-_O_-α-L-rhamnopyranosyl (1→2)-β-D-glucoside (I) from ethyl acetate fraction of leaves. Isolation of 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein) (III) and 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol) (IV) was achieved from methylene chloride fraction of flowers and stem bark, respectively. Kaempferol 3-_O_-α-L- rhamnoside (II) was isolated from ethyl acetate fraction of leaves besides compound (I).

Key words: *Cassia nodosa*, erythrocyte hemolysis, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, bleomycin, cytotoxic, kaempferol rhamnosyl glucoside, kaempferol rhamnoside, rhein, chrysophanol.

INTRODUCTION

In our research we contributed to the other world researchers in using the vast potential offered by natural resources for discovery and development of new therapeutics. This study focused on *Cassia nodosa* Buch.-Ham. ex Roxb. which is known also as *Cassia javanica* L. var. indochinensis Gagnepain (Quattrocchi, 2000). It is commonly called pink Cassia which is a common ornamental tree belonging to the family Leguminosae (Hickey and King, 1997). It is a perennial tree, 3 to 5 m height and scattered in the India, Pakistan,

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Bangladesh and Burma (Yadav et al., 2012). Traditionally, it is useful in the indigenous medicine, as its pods and leaves showed purgative activity (Rizk and Heiba, 1990). It is used for the treatment of cheloid tumor, ring worms, insect bite and rheumatism (Wealth of India, 1963; Nadkarni and Nadkarni, 1976). Bark of *C. nodosa* is used as one of ingredients in anti-diabetic ayurvedic formulation (Joshi et al., 2007). Leaves are proved to be active against *Herpes simplex* infection (Cheng et al., 2006). The presence of anti-diabetic phytochemicals of *C. nodosa* had been noticed. It showed hypoglycemic activity (Kumavat et al., 2012).

The present study has traced detailed biological assessment of variant organs extracts, fractions and some pure constituents to discover if this plant has a significant activity or not. Anti-oxidant, anti-hemolytic, DNA protective and cytotoxic activity against different cancer cell lines types were investigated for the first time. Pure compounds were isolated form leaves, stem bark and flowers. Kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucoside (I) was isolated for the first time, beside that three known constituents; kaempferol rhamnoside (II), 4, 5-dihydroxyanthraqiunone-2-carboxylic acid (rhein) (III) and 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol) (IV) were identified spectrophotometrically (Scheme 1).

MATERIALS AND METHODS

Plant

Leaves, flowers and stem bark were obtained from a house garden in a village in El-Dakahlia governorate. *C. nodosa* was kindly identified by Prof. Dr. Mohammed Ibrahim Fotoh, Professor of Ornamental Horticulture and Landscape Design, Faculty of Agriculture, Tanta University, Tanta, Egypt.

Extraction

The different plant organs (leaves, flowers and stem bark) were air dried and powdered. Cold maceration at room temperature of each powdered organ (1 kg) was carried out in methanol 95% till exhaustion. Different extracts were concentrated under vacuum. Fractionation of methanolic extracts was done using petroleum ether, methylene chloride, ethyl acetate and n-butanol.

General experimental procedures

Concentrated methanolic extracts and their different fractions were obtained at a temperature not exceeding 50°C, using rotary flask evaporator.

Chromatographic materials

Thin layered chromatography (TLC) screening was carried out via pre-coated TLC sheets of 20 × 20 cm GF_{254} Merck. Observation was achieved using Camag UV lamp at 254 and 366 nm. Column chromatography was performed on silica gel 60, E Merck and reversed phase silica C-18, Sigma Chemical Co. Methanol and water for high performance liquid chromatography (HPLC) were purchased from Fischer Scientific UK limited. They were used for ODS column chromatography.

Antioxidant assays

These were performed using different reagents: 2,2`-azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid) ABTS, $MnO_2$, phosphate buffer solution, DNA (Calf Thymus type1), bleomycin sulphate, thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), L-ascorbic acid, 2,2`-azo-bis-(2-amidinopropane) dihyrochloride (AAPH), $FeCl_3$ and HCl. All reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Cytotoxic assay

Four cell lines (WI-38, VERO, MCF-7 and HepG2) were obtained from the American Type Culture Collection (ATTC), RPMI-1640 and fetal calf serum (FCS) were purchased from GIBCO®, dimethyl sulfoxide (DMSO), (4,5- Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) (MTT), sodium dodecyl- sulfonate, 5-Fluorouracil (5-FU), Sigma Chemical Co. St. Louis, MO.

Apparatus

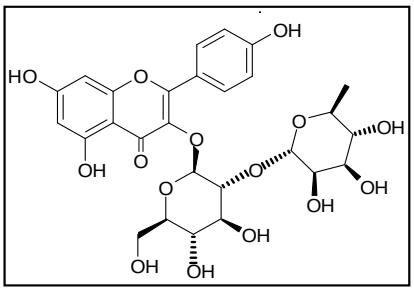
Infrared (IR) spectra were recorded on Nexus 670 fourier transform infrared (FTIR) spectrophotometer. UV spectra were recorded on Shimadzu Spectrophotometer. ^{1}H-(500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on NMR Jeol ECA Spectrophotometer. ELISA Processor II Microplate Reader was used in cytotoxic assessment.

Biological assessment of different extracts and some pure isolated compounds

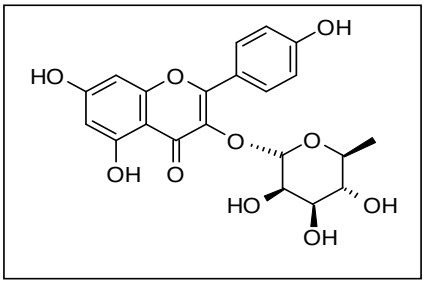
Different methanolic extracts of flowers, leaves and stem bark and their fractions (petroleum ether, methylene chloride, ethyl acetate, n-butanol) were assessed as anti-oxidant, anti-hemolytic, DNA protective and cytotoxic agent. Some pure compounds were also evaluated biologically.

Anti-oxidant assays

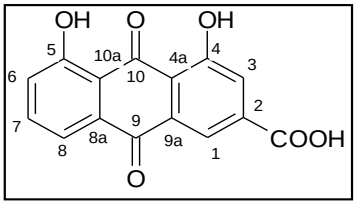
2,2`-azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) screening anti-oxidant assay: The method of Lissi et al. (1999) was adopted for the determination of ABTS activity of pure compounds and different extracts. Breifely, the method was performed as follows: For each of the investigated sample, 2 ml of ABTS solution (60 µM) was added to 3 ml MnO_2 solution (25 mg/ml), all prepared in 5 ml aqueous phosphate buffer solution (pH = 7; 0.1 M). The mixture was shaken, centrifuged, filtered and the absorbance of the resulting green-blue solution (ABTS radical solution) at λ_{max} 734 nm was adjusted to approximately 0.5. Then, 50 µl of 2 mM solution of the tested compound in spectroscopic grade MeOH/phosphate buffer (1:1) was added. The absorbance was measured and the reduction in colour intensity was expressed as inhibition percentage. L-ascorbic acid was used as standard antioxidant (positive control). Blank sample was run without ABTS and using MeOH/phosphate buffer (1:1) instead of tested compounds. Negative control was run with ABTS and MeOH/phosphate



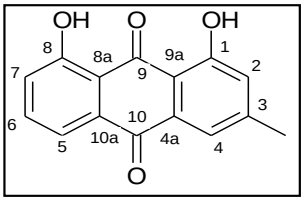
Compound (I)



Compound (II)



Compound (III)



Compound (IV)

Scheme 1. Structures of compounds I to IV.

buffer (1: 1) only. The activities of the samples were evaluated by comparison with a control of ABTS solution and positive control (L-ascorbic acid). Each sample was measured in triplicate and averaged. This activity is given as percentage $ABTS^+$ scavenging that is calculated by the following formula:

$ABTS^+$ scavenging activity (%) = [Ac – As / Ac] × 100

Where A_C is the absorbance value of the control and A_S is the absorbance value of the added samples test solution. Values are means of 3 replicates ± standard deviation (SD) and significant difference at $P < 0.05$ by Student's test.

Erythrocyte hemolysis screening assay: In this method (Malagoli, 2007) blood was obtained from rats by cardiac puncture and collected in heparinized tubes. Erythrocytes were separated from plasma and the buffy coat was washed three times with 10 volumes of 0.15 M NaCl. During the last wash, the erythrocytes were centrifuged for 10 min to obtain a constantly packed cell preparation. Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system. A 10% suspension of erythrocytes in phosphate buffered solution pH 7.4 (PBS) was added to the same volume of 200 mM AAPH solution in PBS containing samples to be tested at different concentrations (stock solution of 1 mg/ml). The reaction mixture was shaken gently while being incubated at 37°C for 2 h. The reaction mixture was then removed, diluted with eight volumes of PBS and centrifuged for 10 min. The absorbance of the supernatant was read at 540 nm. Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis and the absorbance of the supernatant obtained after centrifugation was measured at 540 nm. The data percentage hemolysis was expressed as mean of three replicates. Results were compared to L-ascorbic acid as a positive control.

Bleomycin dependent DNA damage: The reaction mixtures contained in a final volume of 1 ml, with the following reagents at the final concentrations stated: DNA (0.2 mg/ml), bleomycin (0.05 mg/ml), $FeCl_3$ (0.025 mM), magnesium chloride (5 mM), KH_2PO_4-KOH buffer (pH 7.0, 30 mM), L-ascorbic acid (0.24 mM) and the fractions tested compound diluted in MeOH to give a concentration of 0.1 mg/ml. The reaction mixtures were incubated in a water-bath at 37°C for 1 h. At the end of the incubation period, 0.1 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA) was added to stop the reaction (the iron-EDTA complex is unreactive in the bleomycin assay). DNA damage was assessed by adding 1 ml 1% (w/v) thiobarbituric acid (TBA) and 1 ml 25% (v/v) hydrochloric acid (HCl) followed by heating in a water-bath maintained at 80°C for 15 min. The chromogen formed was extracted into butan-l-ol and the absorbance was measured at 532 nm (Aeschbach et al., 1994).

2-Cytotoxic anti-tumor activity against four different cell lines: Four cell lines (WI-38, VERO, MCF-7 and HepG2) were obtained from the American Type Culture Collection (ATTC) and cultured in RPMI1640, supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin sulfate, 100 µg/mL) at 37°C, in an atmosphere of 95% air and 5% CO_2 under humidified condition. A stock solution (10 µM) of samples was prepared in dimethylsulfoxide (DMSO) and diluted with various concentrations with serum-free culture medium. The *in vitro* antitumor activity of different extracts, pure compounds and 5-Fluorouracil (5-FU) were determined by MTT assay method. Exponentially growing different cell lines (4000/well) were seeded in 96-well plates and treated with different concentrations of samples for 48 h and then MTT (1 mg/ml, 100 µl) was added. After incubation for 4 h at 37°C, the crystals of viable cells were dissolved overnight with SDS (sodium dodecylsulfonate, 10%, 100 µl) in each well. The absorbance spectra were measured on an enzyme linked immunosorbent assay (ELISA) Processor II Microplate Reader at a wavelength of 570 nm. The percentage of cytotoxicity was defined with treated and untreated cell lines (Block

et al., 2004; Mosmann, 1983; Wilson, 2000). The 50% antitumor activity dose (IC_{50}) was defined as the concentration of samples that reduced the absorbance of the treated cells by 50%. Classification according to IC_{50} (µg/ml) demonstrate that from 1 to 10 (very strong activity), 11 to 25 (strong activity), 26 to 50 (moderate activity), 51 to 100 (weak activity), 100 to 200 (very weak activity) and above 200 (non cytotoxic) (Ayyad et al., 2012).

Chromatographic separation of pure flavonoids from leaves ethyl acetate fraction: Ethyl acetate dried fraction of leaves (2 g) was chromatographed on a silica gel column (40 × 2 cm) starting with methylene chloride and increasing polarity with methanol. Fractions obtained with (methylene chloride-methanol) (90:10) were rechromatographed on ODS column starting with 100% water for HPLC and decreasing polarity with methanol for HPLC. Compound (I) was eluted using (water:methanol) (80:20) as pure yellow powder. Fractions obtained at eluent ratio (methylene chloride-methanol) (93:7) were pooled, evaporated and rechromatographed on silica gel column (15 × 1.3 cm) to yield yellow powder of compound (II). Identification of pure compounds was achieved by comparing IR, UV, ^{1}H-NMR and ^{13}C-NMR spectral data with those published in the literature.

Chromatographic separation of main anthraquinones from flowers and stem bark methylene chloride fraction: Methylene chloride dried extract of flowers (2 g) was chromatographed on silica gel column (40 × 2 cm) starting with methylene chloride and increasing polarity with methanol. Fractions obtained at eluent ratio (methylene chloride:methanol) (95:5) were pooled, concentrated and rechromatographed on another column of silica gel (15 × 1.3 cm) to provide pure orange powder of compound (III). The previous method of separation was also carried out using 1 g of methylene chloride dried fraction of stem bark. Fractions obtained at eluent ratio (methylene chloride:methanol) (98:2) were pooled and rechromatographed to give pure yellow powder of compound (IV). Identification of pure compounds was also achieved by comparing spectral data with the published data (EI-mass, UV, ^{1}H-NMR and ^{13}C-NMR spectra).

Compound (I): Yellow powder, m.p. 219 to 221°C, freely soluble in methanol, insoluble in petroleum ether, R_f = 0.18 (CH_2Cl_2:CH_3OH) (8:2). IR (KBr) spectrum showed bands at υ_{max} (cm^{-1}): 3464, 2937, 1695, 1608, 1504, 1431, 1371, 1292, 1207. UV λ_{max} (nm): MeOH 266, 348; +NaOMe 274, 325, 395; +$AlCl_3$ 274, 304, 351, 395; $AlCl_3$/HCl 275, 302, 347, 395; +NaOAc 273, 379; +NaOAc/H_3BO_3 266, 347. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR data (125 MHz, CD_3OD) are listed in Tables 1 and 2, respectively.

Compound (II): Yellow powder, m.p. 152 to 153°C, freely soluble in methanol and ethyl acetate, insoluble in petroleum ether, R_f = 0.31 (CH_2Cl_2:CH_3OH:formic acid) (8.5:1.5:0.1) IR (KBr) spectrum showed bands at υ_{max} (cm^{-1}): 3433, 2926, 1655, 1609, 1501, 1454, 1367, 1269, 1209. UV λ_{max} (nm): MeOH 265, 341; +NaOMe 273, 324, 389; +$AlCl_3$ 273, 303, 346, 393; $AlCl_3$/HCl 274, 302, 342, 391; +NaOAc 273, 375; +NaOAc/H_3BO_3 265, 342. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) data are shown in Tables 1 and 2.

Compound (III): Orange powder, m.p. 320 to 322°C, soluble in methanol and chloroform, R_f = 0.25 (CH_2Cl_2:CH_3OH) (9:1). EIMS m/z (rel. int.): 283 $[M]^+$ (9.2), 98 (11), 81 (19.3), 78 (74), 63 (100), 45 (25.5), 28 (62), 18 (45.6). UV λ_{max} (nm): MeOH 430, 232; +NaOH504, 484. ^{1}H-NMR (500 MHz, DMSO-d_6) and ^{13}C-NMR (125 MHz, DMSO-d_6) data are listed in Tables 3 and 4.

Compound (IV): Yellow powder, m.p. 198 to 200°C, freely soluble in chloroform and ether, insoluble in petroleum ether, R_f = 0.91(CH_2Cl_2:CH_3OH) (9:1). EIMS m/z (rel. int.): 254 $[M]^+$ (100), 226 (16.5), 197 (11.2), 181 (3.8), 169 (5), 153 (2.7), 141 (6.1), 127 (4.5), 115 (7.4), 78 (54.7), 63 (71.4). UV λ_{max} (nm): MeOH 429, 287, 256, 224; +NaOH 505, 285. ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) data are shown in Tables 3 and 4.

RESULTS

The $ABTS^+$ scavenging activity of different extracts and pure compounds are demonstrated in Figure 1. Rhein showed the best activity by 74.8% inhibition. Chrysophanol and kaempferol rhamnoside showed activity with average percentage of inhibition of 61.28 and 51.21%, respectively. The anti-hemolytic antioxidant assay results are shown in Figure 2. It was found that chrysophanol exhibited the best anti-hemolytic activity by 3.28% of hemolysis at 0.1 mg/ml, which was better than L-ascorbic acid (3.75%). Kaempferol rhamnoside and rhein also showed anti-hemolytic activity by 4.21 and 5% of hemolysis. The DNA protective activity results are displayed in Figure 3. The absorbance of chrysophanol was 0.069 for sample concentration of 0.1 mg/ml. It showed best protective activity which exceeded the positive control itself (absorbance = 0.47). Kaempferol rhamnoside and rhein exhibited high activity at absorbance of 0.38 and 0.433, respectively. The different extracts unfortunately were inactive in $ABTS^+$ scavenging, antihemolytic, DNA protection assays. This needs more investigation to have the answer. The cytotoxic activity against the different cell lines of VERO, MCF-7, WI-38 and HepG2 are displayed in Figures 4 to 7. It was found that the methanolic extract of stem bark reduced the viability of VERO and MCF-7 cells with IC_{50} = 20.5 and 20.2 µg/ml, respectively. This indicated a strong activity according to the classification. Also, petroleum ether fraction of leaves and flowers with IC_{50} = 25.8 and 29.1 µg/ml showed strong and moderate activity, respectively. Rhein displayed moderate activity with IC_{50} = 26.3 and 28.3 µg/ml, respectively. The petroleum ether fraction of leaves with IC_{50} = 27.3 and 22.4 µg/ml exhibited moderate and strong activity against WI-38 and HepG2 cells, respectively. Rhein showed moderate and strong activity with IC_{50} = 28.4 and 23.7 µg/ml, respectively. Also methylene chloride fraction of leaves showed moderate activity against VERO, MCF-7 and WI-38 cell lines while displayed strong activity against HepG2 cell line.

DISCUSSION

Cancer is a leading cause of death all over the world. Although the etiology of the cancer are many, free radicals play a major role for the pathophysiological processes (Soobrattee et al., 2006). $ABTS^+$ scavenging,

Table 1. ^{1}H-NMR of compound I and II.

#Hydrogen atom	Compound (I) 500 MHz - CD_3OD	#Hydrogen atom	Compound (II) 500 MHz - CD_3OD
OH-5	12.59	H-2`,6`	7.74 (2H, d, *J*=8.4 Hz)
H-2`,6`	8.03 (2H, d, *J*= 8.4 Hz)	H- 3`,5`	6.90 (2H, d, *J*=8.4 Hz)
H-3`,5`	6.86 (2H, d, *J*= 8.4 Hz))	H-6	6.36 (1H, d, *J*=1.5 Hz)
H-6	6. 36 (1H, d, *J*= 1.5 Hz)	H-8	6.18 (1H, d, *J*=2.3 Hz)
H-8	6.16 (1H, d, *J*= 1.55 Hz)	Rh-H-1	5.35 (1H, s)
(H-1) glucose	5.71 (1H, d, *J*= 7.65 Hz)	Rh-CH_3	0.90 (3H, d, *J*=5.53 Hz)
(H-1) rhamnose	4.43 (1H, d, *J*=1.1 Hz)	-	-
CH_3 rhamnose	0.93 (3H, d, *J*= 6.15 Hz)	-	-

Table 2. ^{13}C-NMR of compounds I and II.

#Carbon	Compound (I) (125 MHz - CD_3OD)	#Carbon	Compound (II) (125 MHz - CD_3OD)
C-2	157.0	C-2	157.2
C-3	133.0	C-3	134.8
C-4	178.0	C-4	178.2
C-5	159.9	C-5	157.9
C-6	98.3	C-6	98.5
C-7	164.7	C-7	164.5
C-8	93.2	C-8	93.4
C-9	157.1	C-9	160.2
C-10	104.5	C-10	104.5
C-1`	121.7	C-1`	121.2
C-2`, 6`	130.7	C-2`,6`	130.5
C-3`, 5`	114.7	C-3`,5`	115.1
C-4`	161.8	C-4`	161.8
C-1``(glucose)	101.2	C-1``(rhamnose)	102.1
C-2``	78.7	C-2``	70.71
C-3``	77.0	C-3``	70.76
C-4``	71.0	C-4``	71.8
C-5``	77.6	C-5``	70.5
C-6``	61.2	C-6``	16.3
C-1```(rhamnose)	98.8	-	-
C-2```	70.4	-	-
C-3```	70.9	-	-
C-4```	72.6	-	-
C-5```	68.5	-	-
C-6```	16.1	-	-

erythrocyte hemolytic and bleomycin dependant DNA damage assays were performed because compounds possessing potent antioxidant and anticancer activity may not be useful in pharmacological preparations if they possess hemolytic effect or other side effects. Therefore, a search for compounds that can reduce the harmful side effects of anticancer drugs in normal tissues is necessary (Sun and Peng, 2008). In addition, the different anti-oxidant assays also may reveal some information about the mechanism of cytotoxicity. It was found that pure compounds had a significant anti-oxidant effect comparable to crude extracts and L-ascorbic acid. Rhein was the best $ABTS^{+}$ scavenging agent among the other pure compounds. Chrysophanol displayed the most significant effect. It showed the highest activity as anti-hemolytic and DNA protective agent, which exceeded L-ascorbic acid

Table 3. ^{1}H-NMR of compound III, IV

#Hydrogen atom	Compound (III) 500 MHz - DMSO,d_6	#Hydrogen atom	Compound (IV) 500 MHz - $CDCl_3$
H-3	7.78 (1H, br s)	CH_3	2.46 (3H, s)
H-6	7.33 (1H, d, *J*=8.4 Hz)	H-2	7.1 (1H, br s)
H-1	8.05 (1H, br s)	H-7	7.28 (1H, d, *J*=8.4Hz)
H-7	7.67 (1H, t, *J*=8.4 Hz)	H-4	7.62 (1H, br s)
H-8	7.75 (1H, d, *J*=8.4 Hz)	H-6	7.67 (1H, d, *J*=7.65 Hz)
OH	11.84 (1H, br s)	H-5	7.81 (1H,d, *J*=7.65 Hz)
-	-	OH-1	12.02 (s)
-	-	OH-8	12.13 (s)

Table 4. ^{13}C-NMR of compounds III and IV.

#Carbon	Compound (III) (125 MHz – DMSO,d_6)	#Carbon	Compound (IV) (125 MHz – $CDCl_3$)
C-4	161.5	C-1	162.5
C-3	124.6	C-2	124.4
C-2	134.2	C-3	149.4
C-1	119.9	C-4	121.4
C-8	125.1	C-5	124.6
C-7	138.1	C-6	137.0
C-6	119.1	C-7	120.0
C-5	161.9	C-8	162.8
C-10	191.7	C-9	192.6
C-9	181.4	C-10	182.0
C-11	165.9	(CH_3)	22.3
C-9a	133.6	C-4a	133.7
C-10a	116.6	C-8a	115.9
C-4a	119.2	C-9a	113.8
C-8a	138.6	C-10a	133.3

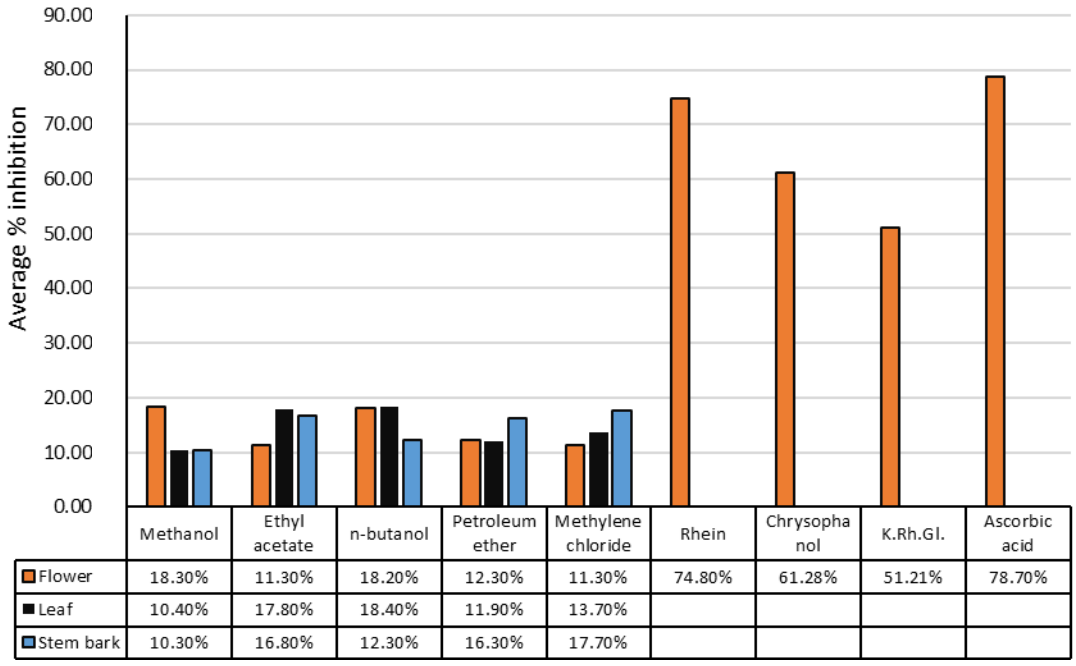
activity (+ve control). Damage to DNA in the presence of a bleomycin-Fe complex has been adopted as a sensitive and specific method to examine potential pro-oxidant agents. If the samples are able to reduce the bleomycin-Fe^{3}+ to bleomycin-Fe^{2}+, DNA degradation in this system would be stimulated, resulting in a positive test for pro-oxidant activity (Gutteridge et al., 1981). Chrysophanol had the highest ability to protect DNA from the induced damage by bleomycin.

Rhein and chrysophanol displayed a significant cytotoxic activity against different cell lines, especially rhein. It is interesting to notice that the high cytotoxic activity of anthraquinones in this study was in agreement with the fact that many herbal formulas-containing anthraquinones were successfully used for treatment of cancer. The most extensively studied anthraquinones, emodin, aloe emodin and rhein have been reported to inhibit proliferation of breast, lung, cervical, colorectal and prostate cancer cells (Cha et al., 2005; Chang et al., 1996; Kuo et al., 1997; Zhang et al., 1995), which is coincident with the results of this study. Several mechanisms have been proposed to explain this anticancer activity, including the intercalation of DNA (Hsiao et al., 2008), inhibition of DNA topoisomerase II (Perchellet et al., 2000), production of free radicals and subsequent cleavage of DNA (Fisher et al., 1992).

Methanolic extract of stem bark, petroleum ether fraction of leaves and flowers methanolic extract and methylene chloride fraction of leaves methanolic extract showed strong to moderate cytotoxic activity, which was in agreement with the fact that the plant is useful traditionally in the indigenous medicine as its leaves and pods had cytotoxic effect (Rizk and Heiba, 1990).

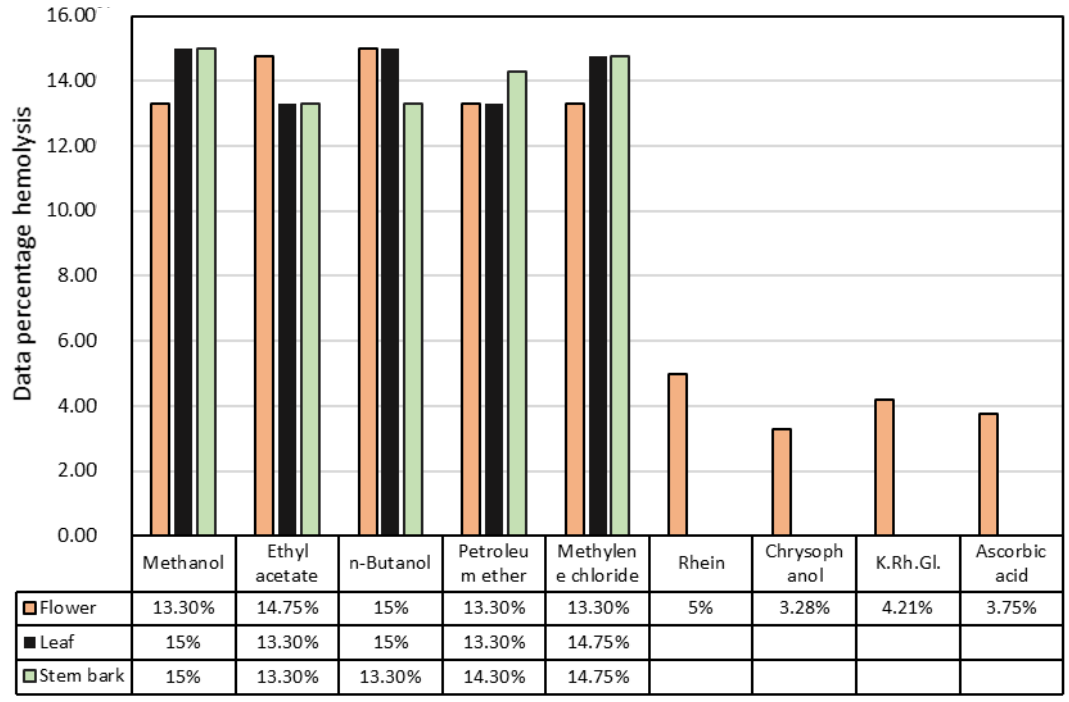
Compound (I)

IR spectrum data revealed that signals at υ_{max} (cm^{-1}) = 3464 is for OH group, 2937 is for aliphatic CH_3 stretching, 1695 is for C=O conjugated, 1608 is for C=C stretching



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	Ascorbic acid
Flower	18.30%	11.30%	18.20%	12.30%	11.30%	74.80%	61.28%	51.21%	78.70%
Leaf	10.40%	17.80%	18.40%	11.90%	13.70%				
Stem bark	10.30%	16.80%	12.30%	16.30%	17.70%				

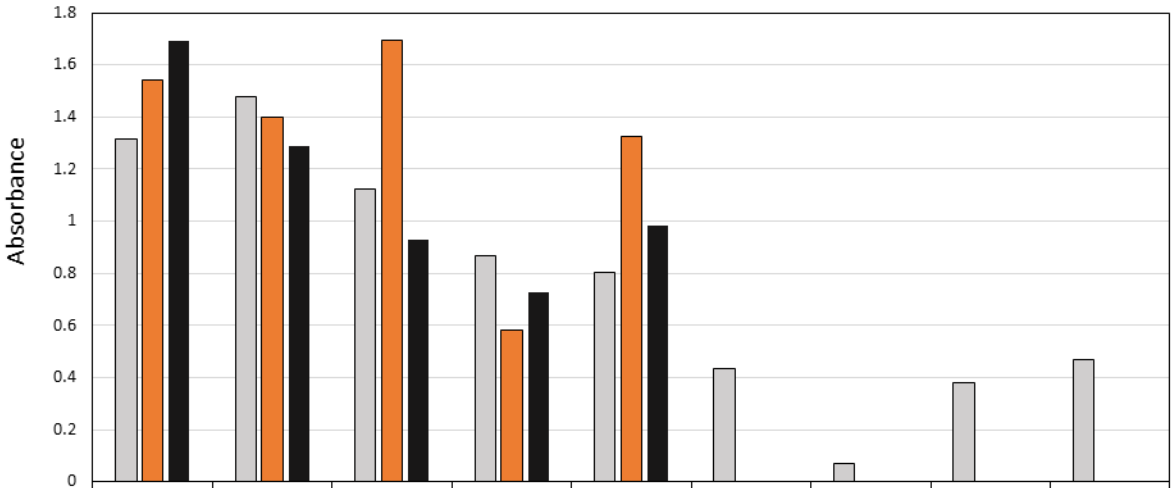
Figure 1. ABTS antioxidant assay.



	Methanol	Ethyl acetate	n-Butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	Ascorbic acid
Flower	13.30%	14.75%	15%	13.30%	13.30%	5%	3.28%	4.21%	3.75%
Leaf	15%	13.30%	15%	13.30%	14.75%				
Stem bark	15%	13.30%	13.30%	14.30%	14.75%				

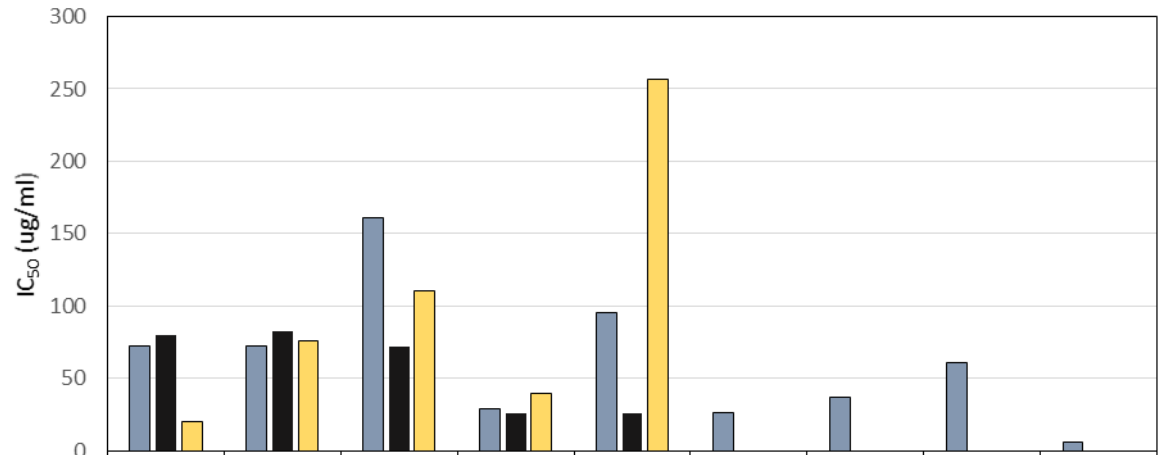
Figure 2. Erythrocyte hemolysis antioxidant assay.

(aromatic), 1431 is for CH_2 bending, 1371 is for CH_3 bending, 1292, 1207 are for C-O. UV spectral data at 348 and 266 nm of compound I was typical of flavonol glycosides substituted at C-3. Shifts obtained with $NaOCH_3$, NaOAC and $AlCl_3$ indicated the presence of free hydroxyl groups at 5, 7, 4` positions. Furthermore,



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	Ascorbic acid
Flower	1.316	1.479	1.124	0.865	0.803	0.433	0.069	0.38	0.47
Leaf	1.54	1.399	1.694	0.584	1.323				
Stem bark	1.692	1.29	0.931	0.727	0.985				

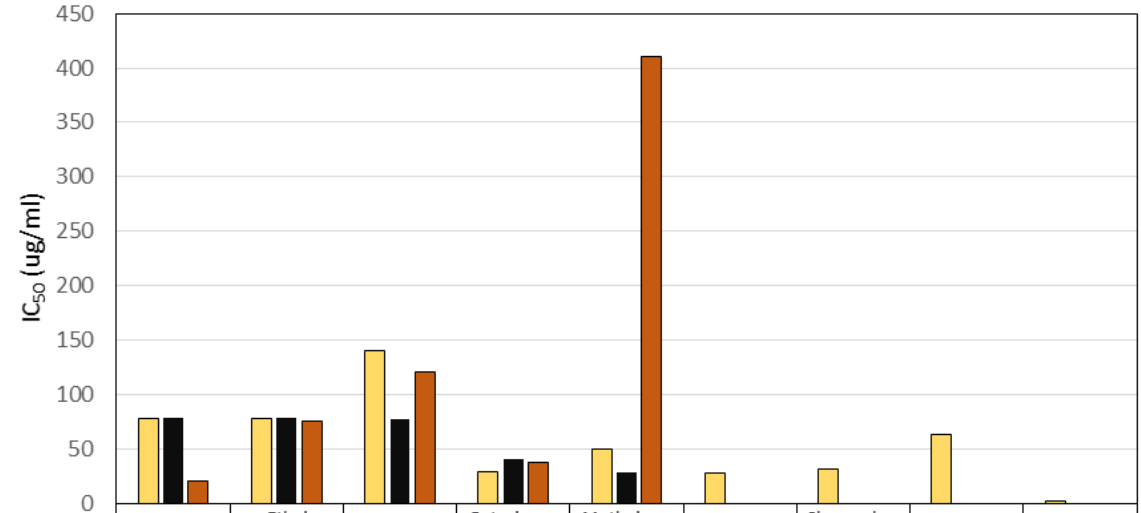
Figure 3. Bleomycin dependent DNA damage assay.



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	5-FU
Flower	72.4	72.4	160.6	28.8	95.6	26.3	36.9	60.7	6.5
Leaf	80.3	82.3	72.4	25.8	26.3				
Stem bark	20.5	76.4	110.4	39.6	256.6				

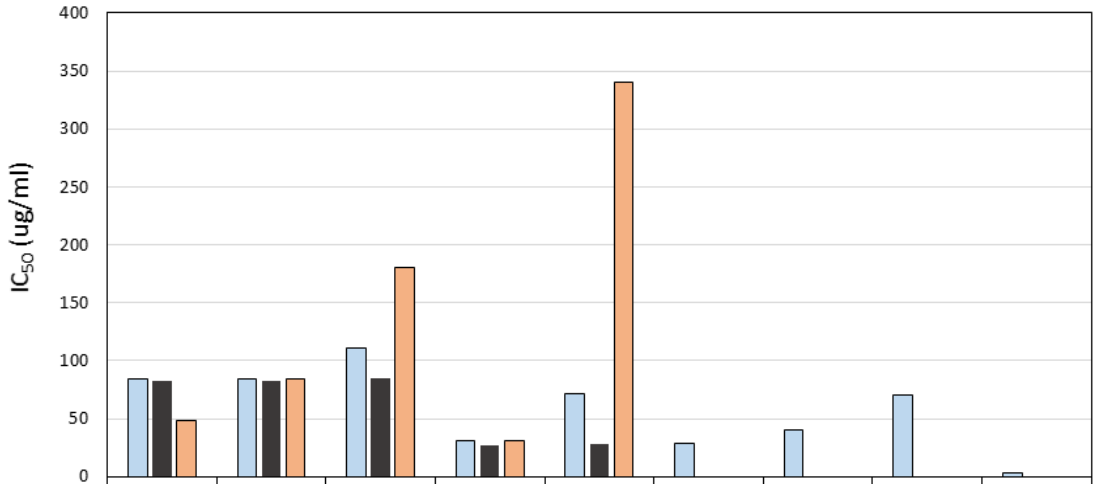
Figure 4. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on VERO cell line.

shifts with $AlCl_3$, $AlCl_3$/HCl and NaOAC/H_3BO_3 did not support the presence of dihydroxyl system (Mabry et al., 1970). Glucose and rhamnose were obtained by acid hydrolysis of compound (I), this confirmed with paper chromatography alongside authentic sugar. ^{1}H-NMR displayed the presence of six aromatic protons ascribed to H6, 8, 2`, 3`, 5`, 6` positions beside two anomeric protons as two doublet signals resonating at δ 5.71, 4.43 ppm with *J* value 7.65, 1.1 Hz, respectively. These two later proton signals can be assigned to anomeric protons of glucose and rhamnose moieties, respectively. The ^{13}C-NMR spectral data showed the presence of anomeric carbons at δ 101.2, 98.8 ppm for glucose and rhamnose moieties, respectively. Furthermore examination of ^{13}C-



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	5-FU
Flower	77.5	77.5	140.5	29.1	49.9	28.3	31.7	63.7	2.3
Leaf	79.2	78.2	77.5	40.8	28.3				
Stem bark	20.2	75.5	120.5	37.4	410.6				

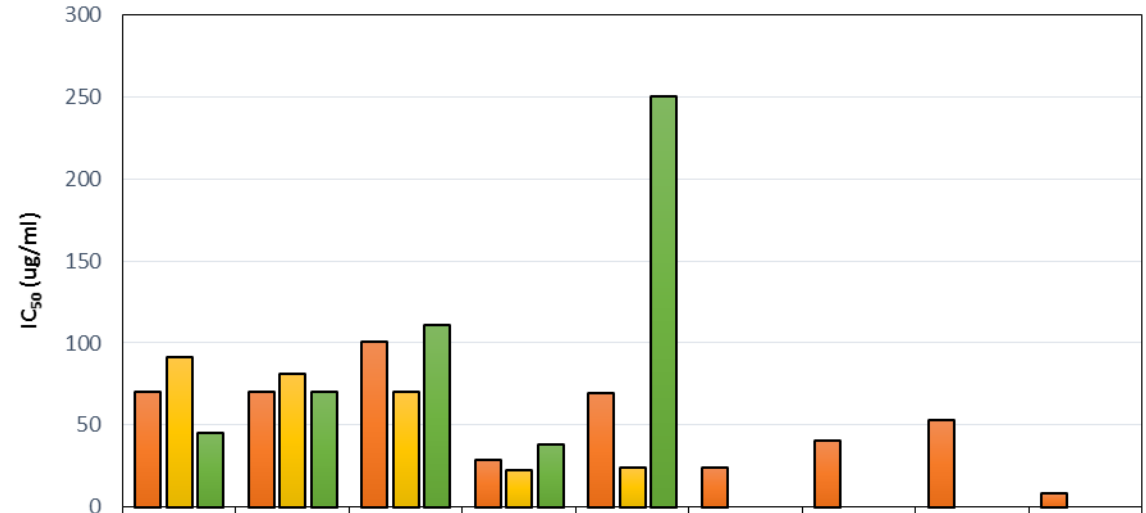
Figure 5. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on MCF- 7 cell line.



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	5-FU
Flower	84.6	84.8	110.6	30.5	71.2	28.4	39.8	70.4	3.2
Leaf	82.4	82.6	84.6	27.3	28.4				
Stem bark	48.2	84.6	180.2	31.1	340.5				

Figure 6. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on WI- 38 cell line.

NMR data indicated rhamno (1→2) glucoside linkage (neohesperidoside). This was evidenced by the lack of shift of C-6`` of glucose, whereas C-2`` signal was de-shielded by 4.5 ppm. Compound (I) was identified as Kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucoside (Kaempferol neohesperidoside) by comparison of spectral data with the published data (Al-Musayeib et al., 2011; Markham and Terani, 1976; Markham et al., 1978;



	methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.RH.Gl.	5-FU
Flower	70.2	70.5	100.7	28.7	69.5	23.7	40.7	52.8	8.6
Leaf	91.2	81.2	70.2	22.4	23.7				
Stem bark	45.1	70.2	111	38.1	250.2				

Figure 7. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on HepG2 cell line.

Takemura et al., 2005).

Compound (II)

IR spectrum data showed that signals at υ_{max} (cm^{-1}) = 3433 is for OH group, 2926 is for aliphatic CH_3 stretching, 1655 is for C=O conjugated, 1609 is for C=C stretching (aromatic), 1454 is for CH_2 bending, 1367 is for CH_3 bending, 1269, 1209 are for C-O. UV spectral properties of Compound (II) were those of typical flavonol glycosides with substituted (OH) group at C-3; the presence of absorption maxima at 341 and 265 nm. Bathochromic shift with NaOMe, NaOAC and $AlCl_3$ indicated for the presence of free hydroxyl groups at 5, 7 and 4` positions. Spectra obtained with $AlCl_3$, $AlCl_3$/HCl and NaOAC/$H_3BO_3$ revealed absence of orthodihydroxyl system at 3` and 4` positions of ring B (Mabry et al., 1970). Rhamnose was obtained by acid hydrolysis, this confirmed with paper chromatography with authentic sugar. $^1$H-NMR spectrum exhibited the presence of six aromatic (H-6, 8, 2`, 3`, 5` and 6`) and one anomeric proton at (δ 5.35 ppm). Protons of methyl group of rhamnose was exhibited at (δ 0.9 ppm, J = 5.53 Hz). ^{13}C-NMR spectrum showed signals at δ 102.1 ppm for anomeric carbon of rhamnose, a significant signal at δ 16.3 ppm for CH_3 of rhamnose. Compound (II) was identified as Kaempferol-3-*O*-α-L-rhamnoside by comparison of spectral data with the published data (Diantini et al., 2012; Matthes et al., 1980).

Compound (III)

EI-Mass spectrum displayed molecular ion peak at 283 $[M]^+$. UV spectrum displayed a bathochromic shift after addition of NaOH. ^{1}H-NMR spectrum showed signals for five aromatic protons (H1, 3, 6, 7 and 8). Phenolic proton signal at (δ 11.84 ppm) was also noticed. ^{13}C-NMR spectrum showed the carbonyl carbons at (δ 165.9 ppm), (δ 191.7 ppm) C-10, (δ 181.4 ppm) C-9, the higher δ value for C-10 is due to strong intramolecular hydrogen bonding with hydroxyl groups at C-4 and C-5 (Agarwal et al., 1999). Compound (III) was identified as 4, 5-dihydroxyanthraqiunone-2-carboxylic acid (rhein), by comparison of spectral data with the published data (Singh et al., 2005).

Compound (IV)

EI-Mass spectrum displayed the parent ion peak at 254 $[M]^+$ which was at the same time the base peak. Strong peaks at 226 and 197 were due to successive elimination of (CO) group indicating the anthraquinoidal nature of the compound (Agarwal et al., 1999). UV spectrum displayed a bathochromic shift after addition of NaOH. ^{1}H-NMR spectrum showed signals for five aromatic protons (H2, 4,

5, 6 and 7). A three proton singlet at (δ 2.46 ppm) indicated the presence of aromatic methyl in the compound. Two phenolic proton signals at (δ 12.02 and 12.13 ppm) were also noticed.[13]C-NMR spectrum showed the carbonyl carbons at (δ 192.6 ppm) C-9 and (δ 182.0 ppm) C-10, the higher δ value for C-9 is due to strong intramolecular hydrogen bonding with hydroxyl groups at C-4 and C-5 (Agarwal et al., 1999). Compound (IV) was identified as 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol) by comparison of spectral data with the published data (Amatya and Tuladhar, 2005).

Conclusion

In our study findings, it was clear that different crude extracts had no antioxidant, anti-hemolytic and DNA protective activities, on the other hand chrysophanol had potent anti-hemolytic and DNA protective activity which exceeded L-ascorbic acid (positive control). Rhein had the best $ABTS^{+}$ scavenging activity. Cytotoxic assessment revealed that stem bark methanolic extract, methylene chloride, petroleum ether fractions of leaves and petroleum ether fraction of flower displayed a significant cytotoxic activity against different cell lines. Rhein as a hydroxy anthraquinone showed a significant anti-tumor activity against all cancer cell lines, when compared to 5-Florouracil (positive control).

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