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Short Communication

Availability of essential medicines in public hospitals: A study of selected public hospitals in Nakuru County, Kenya

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The aim of this study was to confirm the level of availability of essential medicines in public hospitals in Kenya and the factors leading to this. A cross-section approach was adopted for the research which was conducted across several public hospitals in Nakuru County. Data was collected using questionnaires as the main tool. The findings revealed that majority of the essential medicines that include common antibiotics, common analgesics, antihypertensives, emergency drugs and pediatric formulations were found to be stocked out. Stock outs were caused by poor distribution (91.2%), issues of funding (58%), inappropriate selection (58%), and irrational use (56%) of essential medicines.

Key words: Health system, essential medicines, public health.

INTRODUCTION

The World Health Organisation (WHO) defines essential medicines as medicines that satisfy the needs of majority of the population and therefore should be available at all times, in adequate amounts, in appropriate dosage forms and at a price the individual and community can afford (WHO, 2003). Availability of medicines is commonly cited as the most important element of quality by health care consumers, and the absence of medicines is a key factor in assessing the quality of health services (Chuchu, 2006).

Although considerable progress has been made in the last 25 years, since the WHO introduced the concept of essential medicines in 1977; the benefits have been unequally distributed across the global population (Tettah, 2008). Nearly two-thirds of the world’s population is estimated to have access to full and effective treatments with the medicines they need, and majority of these populations are in Asia and Africa (WHO Report, 2009).

Furthermore, the problem may be worsening, as resistance is developing to key medicines for common diseases such as malaria, tuberculosis, and pneumonia. Even though new medicines are being developed to replace those that are no longer effective, frequently these new medicines are more expensive and may require more stringent supervision to ensure they are properly used (Department for International Development (DFID), 2004).

Kimani (2002) in a forum to address Kenya’s health system argues that improving access to essential medicines, and more broadly to health services, is the
key to tackling ill health and reducing mortality rates throughout the developing world.

CURRENT CHALLENGES IN AVAILABILITY OF ESSENTIAL MEDICINES IN KENYA

Supply system

Reliance on certain supply systems can prove severe for a country’s goal of improving access to essential medicines to its populations especially relying on foreign sources (Chaudhuri et al., 2010). Under Kenya’s drug supply system, health centers receive standard kits containing essential drugs from the Kenya Medical Supplies Agency (KEMSA) which sources from other agencies, but this system has been criticized as too rigid and unable to cope with health facilities’ varied needs (Kimani, 2002). The government is therefore piloting a new “pull” system where drug supply is based on orders from health centers, with the hope that this will improve provision of essential drugs in the quantities required.

Frequent stock outs

A WHO (2009) study in Kenya published in the American Journal of Tropical Medicine and Hygiene found that two years after Artemisinin Combination Therapy (ACT) was introduced as the first-line treatment for malaria, one in four surveyed facilities had none of the four recommended weight-specific ACT treatment packs in stock, while three in four were out of stock of at least one of the packs. The shortages sometimes lasted for several weeks. According to Kimani (2002), the government has been working to improve the efficiency of the national drug supply system.

Funding gap

WHO (2012) reports that Kenya now spends 8.87% of the national health budget on medicines, down from 10% in 2009 to 2010 and 12% less than half the WHO’s recommended 34%. Lu et al. (2011) indicate that in order to achieve the global commitment to ensuring access to essential affordable medicines by 2015, an increase in the spending on medicines in low and middle income countries is required.

Current availability of essential medicines

A study done by Health Action International Africa (HAI, 2010) in Kenya, revealed that essential medicines are available in only 50% of lower level health facilities (dispensaries and health centers) and in about 65% of hospitals in Kenya. Recent essential medicines surveys by theWHO in 39 countries mainly low and low- to middle-income countries, including Kenya, found that while there was wide variation, average availability was 20% in the public sector and 56% in the private sector (WHO, 2010).

Orenge (2012) reports that public health facilities were experiencing an acute shortage of drugs thereby forcing hospitals to use funds meant for development to buy emergency medicine from local pharmacies.

In Nakuru County, data obtained from the pharmaceutical agency indicated that in 2010, provincial, district, and sub-district hospitals in the county registered an average availability of 50% for common classes of medicines, while the lower-level facilities had an average of 60% of essential medicines in stock (MOH, 2010). In 2011, the stocks averaged 53% in hospitals and 60% in lower-level facilities (MOH, 2011). In all instances, two to three essential drugs were out of stock in each of the major classes identified by the researcher. According to county data, the stock outs were widespread throughout the county with minimal differences.

It is against this background that this study [Using a cross-sectional approach to describe the availability of essential medicines in public hospitals (Kothari, 2009)] was conducted, with the purpose of ascertaining the unavailability of essential medicines and the factors that contribute to the availability of essential medicines in Kenya’s public hospitals. The goal of this study was to provide reasonable recommendations for policy makers on how to improve this issue throughout the country.

METHODOLOGY

Data were collected through self administered semi structured questionnaires on sample of 44 facilities selected using sample tables (Robert and Darley, 2004). The sampled hospitals in Nakuru County are as follows:

1. Rift Valley PGH
2. Rongai HC
3. Naivasha DH
4. Molo DH
5. Bahati DH
6. Gilgil SDH
7. Njoro HC
8. Subukia HC
9. Elburgon SDH
10. Langa Langa HC
11. Olenguruone SDH
12. Maela HC
13. Kuresoi HC
14. Mbogo-Ini HC
15. Upper Solai HC
16. Kamara HC
17. Engashura HC
18. Mauche HC
19. Keringet HC
20. Engashura HC
21. Mauche HC
22. Keringet HC
23. Mau Narok HC
24. Ndundori HC
25. Kabazi Health Centre
26. Kabatini Health Centre
27. Kapkures Health Centre
28. Lanet Health Centre

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DISCUSSION

The most out of stock drugs was indicated to be analgesics (91% of the respondents indicate that 1 to 3 classes were out of stock) (Figure 1). 56% of the respondents indicate that out of stock have been experienced in 1 to 3 classes of antidiabetic drugs in the last 6 months, and 33% of the respondents indicate that they have experienced out of stock in more than 5 classes of the antidiabetic drugs. Figure 1 also shows that 75% of the respondents indicated that they have experienced out of stock in 1 to 3 classes of common antibiotics in the last 6 months with 21% saying that they have also been out of stock in 3 to 5 classes of drugs.

Antiretroviral (ARV) and antimalarial drugs have experienced low stocking, whereby 68% of the respondents indicated that none of the classes of ARVs has been out of stock in the last 6 months and 21% saying that they have experienced out of stock in 1 to 3 classes. 60% of the respondents indicated that none of the classes of antimalarial drugs has been out of stock, while 39% indicated that they have experienced out of stock in 1 to 3 classes of the drugs.

Out of stock have also been experienced in the last 6 months in antihypertensive drugs, emergency drugs and pediatric formulations by 48, 84 and 88% respondents, respectively. The data (Figure 3) also shows that 16% of the respondents indicated that out of stock have not been experienced in any of the classes of emergency drugs in the last 6 months.

This study found that majority of the essential medicines that include common antibiotics, common analgesics, antihypertensives, emergency drugs and pediatric formulations are stocked out up to a period of a month on an average of 3 classes of the drugs.

Antimalarial and ARV drugs are some of the essential drugs that are less stocked out; however, they too have between 1 and 2 months stock out periods (Figure 2). 63% of the respondents indicated that poor distribution is a factor that causes stock out of essential medicines in public hospitals.

The causal factor that respondents felt most strongly about was inadequate funding (Table 1). Funding of public hospitals comes from the government; therefore, it is clear that the government allocations are not adequate for sustaining quality healthcare through the continuous provision of medicine. Chuchu (2006) asserts that low budgetary allocation to support the Medium Term Procurement Plan (MTPP) was found to be a significant contributor to drug shortages at public health facilities.

Inappropriate selection of medicines is another factor leading to stock outs of essential medicines in public hospitals. The selection of the medicines usually takes place in the procurement stage of the supply chain involving the pharmacists and hospital management team. The responsibility of the pharmaceuticals is often held by the pharmacists, clinical officers, or nurses. In most of the hospitals in this study, a Medicines and Therapeutic Committee did not exist and this hindered the use of best practices in medicine management. According to the WHO Essential Medicine List (EML, 2010), rational medicines selection processes should be in use, based
<table>
<thead>
<tr>
<th>Opinion</th>
<th>Funding</th>
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<td>21.1</td>
<td>21.1</td>
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<td>10.5</td>
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*Multiple responses were allowed on questionnaire.*

![Figure 2](image_url) Number of anti-malarial and ARV drugs O/S. O/S: Out of stock.

![Figure 3](image_url) Number of antihypertensive, emergency and paediatric formulations drugs O/S. O/S: Out of stock.
on national or local essential drug lists and treatment guidelines. The last factor identified was the irrational use of medicines, which greatly hinders the hospitals’ goal of continuous availability of essential medicines. According to Management Sciences for Health (2011), mechanisms at the national and health-facility levels for promoting appropriate use of medicines are weak. Although, the third edition of the National Standard Clinical Guidelines has recently been published and Standard Clinical Guidelines (SCGs) for vertical programs exist, the mechanisms which are responsible for ensuring compliance to these SCGs, such as Medicines and Therapeutic Committees (MTCs), are either weak or not functional in most facilities and must be strengthened and empowered to perform their duties.

The findings of this study confirm that the four factors affecting the availability of essential medicines in public hospitals in Kenya are inappropriate selection, poor distribution, inadequate funding, and irrational use. The following are the author’s recommendations for policymakers:

**Inappropriate selection**

Officers in charge of procurement in public hospitals are sometimes doing a poor job of selecting medicines and are not necessarily following the WHO EML (2010) rational medicines selection processes. Strict use of WHO EML and involvement of users in selection processes is therefore recommended. Also, Medicines and Therapeutic committees should be empowered and involved in medicine selection.

**Poor distribution**

The drug supplier KEMSA is not doing an adequate job of supplying medications, resulting in extended lead times and poor communication between KEMSA and the hospitals, which also complicates the planning process.

Establishment of an elaborate information management system to enhance communication and operations is thus recommended.

**Inadequate funding**

Government allocation for the purchase of medicine and further budgetary allocations by the hospitals are inadequate to ensure continuous availability of essential medicines. The government should commit itself to providing adequate funds to health services, which in turn will increase allocation for the purchase of essential medicines.

**Irrational use**

This is exacerbated by the lack of sufficient training of prescribers in the hospitals and the absence of Medicines and Therapeutic Committees in most hospitals, coupled with limited use of standard treatment guidelines.

Strict adherence to essential medicines lists and standard treatment guidelines is needed to improve selection and appropriate medicine usage.

**ACKNOWLEDGEMENTS**

The authors wish to acknowledge the contributions of Musa Oluooh and Henry M. Macharia in preparing this article.

**Competing Interests**

The authors hereby declare that there were no competing interests.

**REFERENCES**


Full Length Research Paper

Effect of *Cymbopogon proximus* (Mahareb) on ethylene glycol-induced nephrolithiasis in rats

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Nephrolithiasis is a common, painful, costly and recurrent disease, the management of which remains to be an enigma. Phytotherapeutics could be useful as either alternative or complementary therapies in the management of nephrolithiasis. This study was designed to test the efficacy of *Cymbopogon proximus* (CP), a traditional Sudanese plant commonly known as “Mahareb”, in preventing ethylene glycol-induced nephrolithiasis in rats. Thirty male Wistar albino rats were divided randomly into 3 groups of 10. Group 1 was the normal control. Group 2 (stone group) had free access to drinking water containing 0.75% ethylene glycol (EG) and 2% ammonium chloride (AC). Group 3 (test group) was treated as group 2 and was simultaneously injected with *C. proximus* 5% aqueous extract at a dose of 1.5 ml/100 g body weight/day for 10 days. At the end of the treatment period, serum levels of creatinine, blood urea nitrogen (BUN), calcium and phosphorous were determined; measurements of kidney calcium levels were also performed and kidney histopathological examinations were done. The stone group had the highest levels of serum calcium and BUN as well as the highest kidney calcium level. Large crystal deposits were also seen in this group. The CP treated group showed significantly lower levels of serum calcium, serum BUN and kidney calcium (p<0.01); crystal deposits were not observed in this group. The results obtained suggest that CP has a significant protective effect against ethylene glycol-induced nephrolithiasis in rats.

Key words: Sudanese herbal medicine, *Cymbopogon proximus*, nephrolithiasis, calcium oxalate, ethylene glycol, ammonium chloride, aqueous extract.

INTRODUCTION

Kidney stone disease or nephrolithiasis is a common disease with an increasing incidence (Hiatt and Friedman, 1982). It is characterized by a high rate of recurrence (Uribarri et al., 1989), thus prevention is widely recommended. Calcium oxalate (CaOx) represents up to 80% of analyzed stones (Khan, 1997; Jihong et al., 2007). Currently available options for the treatment or prevention of nephrolithiasis are costly, ineffective in all patients or not without side effects (Atmani et al., 2003; Johri et al., 2010). Alternative treatments have been sought especially from herbal medicines, in conjunction with the resurgence of interest in phytotherapy and medicinal plants, as sources of effective, safe, cheap, and socially accepted treatments (Atmani et al., 2003; Johnston, 1997; Bennett and Jand Brown, 2000). In addition, there are several antiuricritic herbal remedies provided by many

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traditional systems of medicine all over the world (Atmani, 2003; Butterweck and Khan, 2009). Nevertheless, the rationale behind the use of many of these remedies has not been fully established using scientific and objective methods. Therefore, it is highly recommended to explore new drugs coming from herbal medicines for the treatment and prevention of kidney stones and to provide experimental evidence and scientific confirmation to their beneficial effects (Atmani, 2003; Butterweck and Khan, 2009). *Cymbopogon proximus* (Family Poaceae) is a traditional medicinal Sudanese plant commonly known as “Mahareb”, which is used in some parts of Sudan to treat kidney stones. It is intensively used in the folk medicine in Sudan for gout, renal colic, helmenthiasis, diuresis, inflammation of the prostate, and as antipyretic (Khalid et al., 2010; Eltohami, 2012). In the Egyptian folk medicine, it is famous as an effective diuretic and renal antispasmodic (El Tahir and Abdel Kader, 2008; El-Askary et al., 2003; Selim, 2011). A decoction of the entire dried herb has been used for centuries in South Egypt as a diuretic, colic pain killer, aid for removal of small stones from the urinary tract, and antipyretic (Selim, 2011). The plant has been found to possess antispasmodic (Selim, 2011), hypotensive (El Tahir and Abdel Kader, 2008), antiemetic (El Tahir and Abdel Kader, 2008), anticonvulsant (El Tahir and Abdel Kader, 2008), hypoglycemic (Mansour et al., 2002), antioxidant (Selim, 2011), antibacterial (Selim, 2011), fungicidal (Fawzi et al., 2009; El-Assiuty et al., 2006), ovicidal and larvicidal (Bassole et al., 2003) properties. The present study was undertaken to assess the effectiveness of *C. proximus* as a prophylactic agent against experimentally-induced nephrolithiasis in rats.

**MATERIALS AND METHODS**

**Plant**

The plants of *C. proximus* (Hochst. ex A. Rich.) Stapf, were collected and botanically authenticated by an expert in taxonomy and voucher specimens were deposited at the National Herbarium, Medicinal and Aromatic Plants Research Institute, National Centre for Researches, Khartoum, Sudan.

**Preparation of plant extract**

The whole plants were washed, air dried and then milled to a fine powder. The powder was suspended in distilled water at a concentration of 5%. The suspension was kept at continuous mixing for 24 h. After settlement, the supernatant (water soluble extract) was used for animals’ administration.

**Animals**

Thirty male albino Wistar rats weighing 150 ± 5 g were allocated into three groups (I, II, and III) of ten animals for each, they were housed in standard cages at standard laboratory conditions (temperature: 25 ± 2°C), and maintained under a controlled 12 h light/dark cycle. All animals had *ad libitum* access to normal drinking water and standard rat diet. Prior to experiment commencement, animals were left one week for an acclimatization period. Experiments were conducted in accordance with internationally accepted standard guidelines for the use of animals.

**Reagents**

Ethylene glycol (EG; VEB Laborchimie Apolda, Germany), was used to produce hyperoxaluria and consequently the deposition of CaOx crystals in rat kidneys. It was added in drinking water to a final concentration of 0.75% V/V. Ammonium chloride (AC; Elnasr Pharmaceutical Chemistry Co., Egypt) was combined with EG to enhance crystal deposition. It was also added in drinking water to a final concentration of 2% W/V.

**Experimental design**

Animals in group I served as the normal or negative control group and received no treatment throughout the experimental period. Group II animals (positive control group) received the crystal inducing chemicals, 0.75% EG and 2% AC in their drinking water for ten days. Animals in group III (test or preventive regimen group) were treated as group II and in addition they were given simultaneously 5% *C. proximus* aqueous extract daily by gavage at a dose of 1.5 ml/100 g body weight (Fan et al., 1999).

**Assessment of antiurolithic activity**

At the end of the 10 days treatment period, animals were mildly anaesthetized using light ether; blood samples were collected from the retro-orbital region in non-heparinized tubes, centrifuged at 3000 rpm for 15 min. to obtain serum. Samples were then analyzed for creatinine, blood urea nitrogen (BUN), calcium and phosphorous levels. After the collection of blood samples, animals were sacrificed, abdomens opened and kidneys removed. The left kidneys were dried in an oven at 100°C. Each kidney was then weighed and subsequently minced (homogenized) in a beaker containing 7 ml of 0.5 N nitric acid, the mixture was then heated until dissolution was affected and a transparent liquid was obtained, from which the kidney calcium level was determined using flame spectroscopy. The amount of calcium was expressed as µg/g dry kidney ( Touhami et al., 2007). The right kidneys were fixed with 10% neutral buffered formalin and subsequently embedded in paraffin, cut into 4 µm thick sections for slides, and stained with hematoxylin and eosin. The sections were examined under light microscopy by a histopathologist (Figure 1).

**Statistical analysis**

Data were presented as the mean ± standard deviation (SD). One way analysis of variance (ANOVA) was used for comparisons among different groups followed by the post hoc test, Fisher’s least significant difference (LSD) for multiple comparisons. P value < 0.05 was considered to indicate a significant difference. Statistical Package for Social Sciences (SPSS) version 18 was used for this analysis.

**RESULTS**

**Serum analysis**

Serum levels of creatinine and BUN were significantly
elevated in the positive control group as compared to the normal control group; whereas the test group showed lower levels of both creatinine and BUN, approximating normal values. Calcium levels were reduced in the positive control group (6.23 mg/dl) in relation to the negative control group (8.62 mg/dl), while the test group (EG/AC/CP treated) revealed a significant ($P < 0.001$) elevation in calcium levels (7.03 mg/dl) as compared to the positive control group. Serum levels of phosphorous were the highest in the positive control group (8.94 mg/dl), while the test group had the lowest levels (7.25 mg/dl). Differences were significant ($P < 0.001$) among the three groups (Table 1).

### Calcium levels in the kidneys

Calcium levels in the kidneys of rats in the positive control group, were remarkably increased (28.47 µg/dl) compared to levels produced by the negative control rats (7.69 µg/dl). Simultaneous treatment of rats in the test group with the herbal extract significantly reduced kidney calcium contents (9.33 µg/dl) with a probability of chance occurrence ($P < 0.001$).

### Histological examination

Renal cortices and medullas of animals in the positive control group showed marked tubular cell necrosis, tubular atrophy, intraluminal deposits, diffuse interstitial edema, interstitial inflammatory cell infiltrate, and focal fibrosis (Figure 2). Glomerular morphology remained unchanged except that few collapsed glomeruli were seen. Plant treated group showed almost normal histological features, no calcium deposits were seen (Figure 3). These morphological findings were consistent with the data of the left kidney calcium level.

### DISCUSSION

At present, antiurolithic treatments provided by conventional medicine are costly, ineffective in all patients, not well tolerated, or not without side effects (Atmani et al., 2003; Johri et al., 2010). In addition, recurrence is a central problem in urolithiasis with quite high rates (Uribarri et al., 1989; Ruml et al., 1997) and thus prophylactic treatment is highly recommended. Various pharmacological and non pharmacological treatments have been applied for this aim. Although, these preventive measures have improved the medical management of nephrolithiasis and lead to reduction in the rates of stone formation and prolongation of the post surgical stone-free periods (Ruml et al., 1997), stone recurrence can be reduced by only half, and there is so far no satisfactory drug for the treatment of nephrolithiasis, especially for the prevention or the recurrence. Due to the complications of nephrolithiasis treatment along with the worldwide resurgence of interest in medicinal plants and phytotherapy (Johnston, 1997; Bennett and Jand Brown, 2000), there has been a trend to look for alternative antiurolithic treatments from natural sources and to test the antilithic remedies that are found in many traditional systems of medicine all over the world (Atmani et al., 2003; Atmani, 2003; Butterweck and Khan, 2009).

This study was designed to prove the possible antiurolithic property of one of the herbs that are used traditionally for kidney stones, C. proximus. The preventive effects of the plant extract on a calcium oxalate urolithic rat model induced by the ingestion of EG in conjunction with AC were particularly studied. By combining two urinary stone risk factors (hyperoxaluria and hypocitraturia), EG/AC combination is an accelerated nephrolithic by the seventh day of treatment (Khan, 1997; Fan et al., 1999).

Assessment of serum creatinine and blood urea nitrogen (BUN) was carried out to test the renal function, and as markers of glomerular and tubular damage (Thamiliselvan and Menon, 2005). Results revealed significantly higher levels of creatinine and BUN in the EG/AC treated group when compared with the normal control group ($P < 0.001$); in agreement with the results of other studies (Jihong et al., 2007; Touhami, 2007; Thamiliselvan and Menon, 2005; Al-Attar, 2010; Bahuguna et al., 2009). In nephrolithiasis, the glomerular filtration rate (GFR) is reduced due to obstruction of the urinary outflow with stones; as a result waste products particularly nitrogenous compounds such as creatinine,
uric acid and urea accumulate in the blood. In addition, exposure to high levels of oxalate and calcium oxalate crystals is known to induce lipid peroxidation in renal tubular epithelial cells which is associated with the production of free radicals; this will cause damage to the renal tubular membrane surface and is considered to be a prerequisite for the nucleation, attachment, and development of CaOx kidney stones (Thamilselvan and Menon, 2005; Thamilselvan et al., 2000). In this study, renal damage was indicated by the elevated creatinine and BUN (Touhami et al., 2007; Bahuguna et al., 2009).

Two more serum parameters were assessed, calcium and phosphorous. Results revealed that the EG/AC treated group had a lower calcium and a higher phosphorus level in serum as compared to the normal group (P < 0.001). The reduced serum calcium level correlates with an increased urinary calcium excretion and a subsequent deposition of calcium oxalate in the kidney as a consequence of treatment with EG. Similar results were obtained from other studies (Al-Attar, 2010; Rajagopal, 1977; Hiramaya et al., 1993). In contrast, a study by Touhami et al. (2007) showed an elevated serum calcium in the EG/AC treated group; the cause of which was not explained (Touhami, 2007). With regard to phosphorous, the group treated with EG/AC showed a significantly higher serum level in agreement with the findings of other studies; and is explained as being the result of the nephrotoxic effect of glycol (Touhami, 2007; Rajagopal, 1977; Patel et al., 2011). On the other hand, studies by Tsai et al. (2008) and Tsai et al. (2009), have shown a lower level of serum phosphorous, in EG fed rats (Tsai et al., 2008; Tsai, 2009), whereas Al-Attar (2010) obtained insignificant changes.

Levels of calcium in the kidneys were also determined as an evidence of the presence of calcium deposits. Results showed that the EG/AC treated group had about four folds higher renal tissue calcium level than the normal control group; in consistence with previous findings (Jihong et al., 2007; Touhami et al., 2007; Fan et al., 1999; Bahuguna et al, 2009; Mitra et al., 1998; Soundararajan et al., 2006).

For further confirmation, histopathological examinations were performed. Results revealed that rats fed with 0.75% EG plus 2% AC in drinking water for 10 days (positive control group), successfully produced renal deposition of CaOx crystals. Intratubular crystal deposits were seen in both cortex and medulla; in addition, there was marked tubular cell necrosis, tubular atrophy, diffuse interstitial edema, interstitial inflammatory cell infiltrates.
and focal fibrosis; few collapsed glomeruli were also observed (Figure 2). These findings are consistent with the findings of Fan et al. (1999), Jihong et al. (2007) and Touhami et al. (2007) and they provide extra validation to the use of combined EG/AC rat urolithiasis model.

In the group treated with *C. proximus*, 5% aqueous extract simultaneously with EG/AC stone induction, there was a significant (P < 0.001) reduction in serum levels of creatinine and BUN as compared to the EG/AC treated group indicating an improved or almost normal renal function. Serum calcium levels were markedly elevated in this group approximating the value of the normal group, in contrast to the reduced calcium level in the EG/AC group; this suggests that the plant administration caused a reduction in the urinary excretion of calcium which is usually increased after EG ingestion and is eventually followed by the deposition of CaOx crystals in renal tissue, thus the plant by this way may prevent CaOx stone formation. Phosphorus level which was elevated in the EG/AC group, was significantly reduced in the plant treated group even to a lower level than that of the normal group; this indicates a protective action of the plant against glycol nephrotoxicity.

Kidney calcium contents in the CP treated group were significantly reduced (P < 0.001) as compared to calcium levels in the EG/AC only treated group indicating none or minor calcium crystal deposition in the plant treated group.

Histopathology findings in the CP treated group showed almost normal renal architecture. There was no tubular cell necrosis, no tubular lumen dilation, no intraluminal stone, nor diffuse interstitial edema. Only mild interstitial inflammatory cell infiltrates were observed, while the glomerular morphology remained unchanged (Figure 3). These findings when compared with the EG/AC only treated group suggest a potent protective effect of the plant extract against EG/AC induced nephrolithiasis and nephrotoxicity.

From the results of this study, it was supposed *C. proximus* to have a preventive effect against CaOx nephrolithiasis that could be attributed to one or more actions of the plant. First, the plant has a well known, highly reputed diuretic action (Khalid et al., 2010; Eltohami, 2012; El Tahir and Abdel Kader, 2008; El-Askary et al., 2003; Selim, 2011), and as far as renal stones are concerned, an increased urine output is desirable.

**Figure 2.** Kidney paraffin section viewed under light microscope of a rat from the positive control (EG/AC) treated group. (A) The arrow points to intraluminal stone debris (H&E, x100). (B) The arrow points to tubular cell necrosis (H&E, x100). (C) The arrow points to tubular atrophy (H&E, x200). (D) The arrow points to interstitial edema and interstitial inflammatory cell infiltrate (H&E, x200).
so that the concentration of stone forming constituents is kept below the level of supersaturation which is the starting event in the process of stone formation. Secondly, an antilithic effect of the plant can be solely explained in terms of its antioxidant property, due to the increasingly growing body of evidence that suggests a decisive role of hyperoxaluria induced oxidative stress and renal cell injury in the pathogenesis of stone disease (Thamilselvan and Menon, 2005; Thamilselvan et al., 2000). Indeed many antioxidants and free radical scavengers have been studied in this context and were shown to inhibit CaOx crystal deposition by way of improving the tissue antioxidant status (Thamilselvan and Menon, 2005; Selvam, 2002; Grases et al., 2009). In addition, the antiurilithic effect of some herbals was claimed in recent studies to be due to their antioxidant activity rather than a diuretic effect or a change in urinary chemistry (Grases et al., 2009; Itoh et al., 2005; Sailaja et al., 2011; Ashok et al., 2010; Dodoala et al., 2010; Bashir et al., 2010). *C. proximus* was shown in a study by Selim (2011) to possess a strong antioxidant activity; the total antioxidant capacity of *C. proximus*, expressed as the number of equivalents of ascorbic acid, was found to be 48.66±3.1. Methanolic extracts of *C. proximus* also showed a highly effective free radical scavenging in the DPPH assay (Selim, 2011). The plant contains the flavonoids rutin and quercetin (Heiba and Rizk, 1986) which are well known antioxidants, both of which have been explored for antiurilithic activity and were proved to be effective (Park et al., 2008; Ghodasara et al., 2010). Besides the diuretic and antioxidant effects, the plant has a unique antispasmodic property as it produces smooth muscle relaxation while preserving the propulsive movement of the tissue (El-Askary et al., 2003; Selim, 2011), thus it has a further beneficial effect of relieving renal colic pain which is commonly associated with kidney stones, and at the same time aiding in the propulsion of stones especially ureteric ones. The antispasmodic principle of the plant “proximadiol” has been isolated and is already traded for this purpose (Abdel-Azim et al., 2011). Moreover, the potent antibacterial effect of the plant against multiantibiotic-resistant organisms (Selim, 2011) may be useful, as kidney stones are sometimes complicated with urinary tract infections especially if there is obstruction; and conversely infections can be a cause of certain types of stones such as struvite stones and even calcium stones.

Recent evidence suggests a role of intracellular

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Figure 3. Paraffin section of a rat kidney from the *C. proximus* treated group showing no tubular cell necrosis neither intralumenal stone debris, but mild interstitial inflammatory cell infiltrate (H&E, x200).
nanobacteria which are capable of producing a calcium phosphate shell, and thus could serve as nucleation sites for stone formation (Kramer et al., 2000).

REFERENCES


Full Length Research Paper

New activity for old drug: *In vitro* activities of vitamin K₃ and menadione sodium bisulfite against methicillin-resistant *Staphylococcus aureus*

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To discover new lead compounds against methicillin-resistant *Staphylococcus aureus* (MRSA), trimethylhydroquinone, vitamins K₁, K₂, K₃ and menadione sodium bisulfite were targeted for anti-MRSA assay. Their anti-MRSA activities were evaluated by agar diffusion method, and their minimum inhibitory concentrations (MICs) were determined by broth microdilution method. The results showed that trimethylhydroquinone, vitamin K₃ and menadione sodium bisulfite presented obvious anti-MRSA activity, and their MICs against MRSA ATCC 33592 and three clinical MRSA isolates were successively 16 to 32, 8 to 16 and 16 µg/ml. Vitamins K₁ and K₂ showed no anti-MRSA activity when the test discs respectively carried 1024 µg of them. These indicated that the anti-MRSA activity would disappear when the methyl of vitamin K₃ was substituted by alkyl that contained four isopentenyl units, and vitamin K₃ probably has an ancillary effect on the treatment of MRSA infection.

**Key words:** Vitamin K₃, menadione sodium bisulfate, trimethylhydroquinone, methicillin-resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious threat to public health because of its resistance to multiple antibiotics (Payne, 2008; Taubes, 2008). To overcome resistance, various efforts at discovery of novel antibiotics were carried forward, such as classic screening methods, chemical modification of known antimicrobials, improving antimicrobial activity of known compounds, hybrid agents and new targets (Moellering, 2011). Moreover, it is worth noticing that discovering anti-MRSA compounds from known compounds and drugs should be an economical and effective method (Berger, 2007; Chong and Jr Sullivan, 2007).

Base on the structure-activity analysis of anti-MRSA natural products (Saleem et al., 2010; Gibbons, 2004), some phenols and quinones contained hydrophobic groups were deduced to present potential anti-MRSA activity. So, three potential anti-MRSA compounds as trimethylhydroquinone (compound 1) (Figure 1), α-tocopherol and phloroglucinol were selected for anti-MRSA assays. The results showed that compound 1 presented obvious anti-MRSA activity with daptomycin as a positive control, while α-tocopherol and phloroglucinol
showed no activity against MRSA (Data was not reported). Since the skeleton structures of vitamins K₁ (compound 2), K₂ (compound 3), K₃ (compound 4) and menadione sodium bisulfate (compound 5) were a little similar to that of 1 (Figure 1), these four compounds may also show anti-MRSA activities. To prove this and discover new anti-MRSA activity for old drugs, compounds 2 to 5 were further targeted for anti-MRSA assay, and in vitro activities of them against three clinical isolates of MRSA, two reference strains as MRSA ATCC 33592 and methicillin-susceptible S. aureus (MSSA) ATCC 25923 were evaluated.

**MATERIALS AND METHODS**

**Reagents**

Compounds 1, 2, 4 and 5 were purchased form Aladdin Industrial Corporation (Shanghai, China), and compound 3 and daptomycin (Purity of 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds avoided light, and were stored at 4°C. Mueller-Hinton Agar (MHA) and Mueller-Hinton broth (MHB) used for anti-MRSA assay were purchased from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China) and were used for the measurement of anti-MRSA activities. The water used was freshly distilled, deionized and purified with Milli-Q plus equipment (Millipore, Bedford, MA). All other solvents and reagents used were of analytical grade. A top pipette 20 to 200 μl and an eight channels pipette 50 to 300 μl were purchased from Dragon Laboratory Instruments Limited (Beijing, China). Ninety six-well plates were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

**Strains**

Two reference strains as MRSA ATCC 33592 (gentamycin and methicillin-resistant) and MSSA ATCC 25923 were purchased from ATCC (American Type Culture Collection, USA). Three clinical MRSA isolates as MRSA 01, MRSA 02 and MRSA 03 were obtained from Clinical Laboratory of the Second Affiliated Hospital, Sun Yat-sen University, Guangzhou, China (Yuan et al., 2012). These three MRSA isolates were identified by PBP2a Latex Agglutination Kit test, and were respectively resistant to erythromycin and methicillin (MRSA 01), methicillin (MRSA 02) and gentamycin and methicillin (MRSA 03). All these MRSA and MSSA strains were sub-cultured with MHB at 35°C for 24 h prior to anti-MRSA assay.

**Inhibition zones**

The inhibition zone of compounds 1 to 5 against all MRSA strains were performed using agar diffusion method described as Clinical and Laboratory Standards Institute standard with a little modification (CLSI, 2012). Compounds 2 to 4 were respectively dissolved in ethyl acetate to obtain sample solutions with the concentrations of 20.48, 20.48 and 5.12 mg/ml, and compounds 1, 5 and daptomycin were respectively dissolved in purified water to obtain sample solutions with the concentrations of 2.56 mg/ml, 5.12 mg/ml and 64 μg/ml. Daptomycin solution and ethyl acetate were respectively used as a positive control and a solvent control. Diluted inoculums (100 μl, 0.5 of McFarland standard) of MRSA ATCC 33592 and MSSA ATCC 25923 were respectively spread on MHA plates using sterile cotton swab. Next, each sample solution was circularly dropped on a 10 mm diameter disc with pipette and dried in the clean bench until 50 μl of it was carried. After this, each disc was placed on the surface of MHA plates, and then incubated at 35°C for 18 h. All tests were repeated twice, and the test plates stood in dark to avoid the degradation of compounds 1 to 5 in the culture process. The results were described as the diameters (in mm) of inhibition zones, and expressed as mean value ± standard deviation (SD).

**Minimum inhibitory concentrations (MICs)**

The MICs of compounds 1, 4 and 5 against all MRSA strains were determined by broth microdilution method described as CLSI standard with a little modification (CLSI, 2012). The tests were
performed on 96-well plates in duplicate, and daptomycin was used as a positive control. The sample solution of compound 4 was prepared with dimethyl sulphoxide (DMSO) and MHB, and the initial well contained 5% DMSO in the 96-well plate. So, 5% DMSO aqueous solution was used as a solvent control. Briefly, 100 μl MHB was added into each well. 100 μl each compound solution (256 μg/ml) was respectively added into column 1, and was mixed with pipette. Then starting with the concentration of 128 μg/ml for each compound, two-fold dilution was followed. Subsequently, 100 μl diluted inoculum (0.5 of McFarland standard) was added to each well. Finally, the test plates were incubated at 35°C for 24 h, and also stood in dark to avoid the degradation of compounds 1, 4 and 5 in the culture process. The microbial growth was observed by adding 20 μl of 2.0 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). When the microbial growth in the well of solvent and growth controls was good (Color change from yellow to blue purple), the MICs of samples were determined as the lowest concentration where bacterial growth was visibly inhibited (no obvious color change from yellow).

**RESULTS**

As the most important member of vitamin K₂ homologs, compound 3 (menaquinone-4, MK-4) was used as vitamin K₂ in this research. The results (Table 1) indicated that compounds 1, 4 and 5 presented obvious activity against MRSA ATCC 33592 and MSSA ATCC 25923 with respective inhibition zones of 20.3 to 21.3, 30.0 to 30.3 and 29.0 to 30.0 mm, and those for daptomycin were 21.0 to 21.7 mm (the reference for the inhibition zones of 30 μg daptomycin against MSSA ATCC 25923 were 18 to 23 mm). While compounds 2 and 3 showed no anti-MRSA activity. Further, the minimum inhibitory concentrations (MICs) of compounds 1, 4 and 5 against all four MRSA strains were respectively 16 to 32, 8 to 16 and 16 μg/ml, and those of daptomycin were 0.5 to 1 μg/ml (the reference for MICs of daptomycin against MRSA ATCC 33592 was less than 1 μg/ml) (Table 2).

**DISCUSSION**

Vitamin K₂ (compound 2) known as phyloquinone, is biosynthesized by plants. Menaquinone-4 (compound 3, MK-4) was the most important member of vitamin K₂ homologs called menaquinones, has several subtypes, and is biosynthesized by animals and bacteria. While compound 4 is a synthetic form of vitamin K. Considering that compound 4 is insoluble in water, compound 5 is usually its application form. Meanwhile, vitamin K₃ can be converted to vitamin K₂ in vivo (Shearer and Newman, 2008). After the anti-MRSA activity of compound 1 was discovered, their similar skeleton structures to compound 1 deduced that compounds 2 to 5 may also show anti-MRSA activity. Based on these facts, a hypothesis that anti-MRSA compounds would be found everywhere (in plant, animal, human and bacteria) was put forward if compounds 2 to 4 were proved to be active against MRSA. So, the anti-MRSA assays of compounds 2 to 5 were further evaluated with daptomycin used as a positive control. The results showed that the anti-MRSA activity of compound 4 was basically equal to that of compound 5 by comparing their molar concentrations calculated from their MICs, while it was about twofold stronger than that of compound 1. Many new bioactivities of vitamin K₃ (compound 4 or 5) and its derivatives were discovered recently, such as anti-cancer (Matzno et al., 2008; Tomasetti et al., 2012; Tanahashi et al., 2011), anti-Alzheimer's disease (Huy, et al., 2013), anti-allergic and anti-inflammatory effects (Kohli et al., 2011; Chinzei

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Table 1. Diameters of inhibition zones of 1 to 5 against two reference strains (n = 3).

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Daptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA ATCC 33592</td>
<td>20.3 ± 1.2</td>
<td>-**</td>
<td>30.3 ± 1.5</td>
<td>29.0 ± 1.5</td>
<td>21.0 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>MSSA ATCC 25923</td>
<td>21.3 ± 1.5</td>
<td>-</td>
<td>30.0 ± 2.0</td>
<td>30.0 ± 1.7</td>
<td>21.7 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

*The diameter of all discs was 10 mm, and the amount of 1, 2, 3, 4, 5 and daptomycin were respectively 128, 1024, 1024, 256, 256 and 32 μg. **Indicated that there was no inhibition zone.

Table 2. Minimum inhibitory concentrations of 1, 4 and 5 against MRSA strains.

<table>
<thead>
<tr>
<th>MRSA strain</th>
<th>1</th>
<th>4</th>
<th>5</th>
<th>Daptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA ATCC 33592</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>MRSA 01</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>MRSA 02</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>MRSA 03</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>1</td>
</tr>
</tbody>
</table>
et al., 2011). Here, the obvious antibacterial activity especially anti-MRSA activity of vitamin K₃ (compound 4 or 5) was first discovered by us, which showed that vitamin K₃ probably have an ancillary effect on the treatment of MRSA infection except for being used as vitamin K supplement and potential cancer treatment.

Moreover, it is unfortunate to find that compounds 2 and 3 have no anti-MRSA activity when the test discs, respectively carried 1024 µg of them, which showed that the anti-MRSA activity would disappear when the C₂ methyl of compound 4 was substituted by alkyl that contained four isopentenyl units. This raises the following questions: (1) What is the reason? and (2) how do we interpret another obvious and thoughtful fact that vitamin K₃ has obvious toxicity and aforementioned bioactivities, while vitamin K₂ has no toxicity and bioactivities except as a supplement of vitamin K? It maybe very worthy to further research the molecular mechanism and the structure-activity and structure-toxicity relationships involved the alkyl side-chain substituted at C₂ position of vitamin K₃.

Conclusions

Conclusively, in vitro activities of vitamin K₃, menadione sodium bisulfate and trimethylhydroquinone against MRSA were determined. The results indicated that these three compounds presented obvious anti-MRSA activity, and showed that vitamin K₃ or menadione sodium bisulfite probably has an ancillary effect on the treatment of anti-MRSA infection.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

MRSA, Methicillin-resistant Staphylococcus aureus; MIC, minimum inhibitory concentration.

REFERENCES


Pharmacological effect of some fractions obtained from *Sapindus trifoliatus* acting as an antioxidant and against mammary cell proliferation

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To explore the naturally-occurring antioxidants having anticancer properties from plant origin since oxidants play a crucial role in developing various human diseases. The purpose of this research was to explore the antioxidant and anticancer properties of *Sapindus trifoliatus* (ST). The dried leaves of *S. trifoliatus* were ground into coarse powder and were exhaustively extracted with methanol; and the resulting crude methanolic extract (CME) was successively fractionated with petroleum ether, chloroform and ethyl acetate to obtain final extracts of petroleum ether (PEF), chloroform (CHF), ethyl acetate (EAF) and lastly the fractions of aqueous (AQF). Several assays were employed to determine the antioxidant activities, such as, 1,1-diphenyl-2-picrylhydrazine (DPPH) free radical scavenging assay, total antioxidant capacity assay, ferrous reducing antioxidant capacity, hydroxyl radical scavenging assay and lipid peroxidation inhibition assay. The *in vivo* anticancer activity of *S. trifoliatus* was deduced on Ehrlich’s Ascites cell (EAC) induced Swiss albino mice. Majority of the extracts showed strong antioxidant activities related to the standard.

**Key words:** *Sapindus trifoliatus*, Sapindaceae, free radicals, polyphenolics, flavonoids, antioxidant activity, anticancer activity.

**INTRODUCTION**

Here, it is pertinent to mention the oxidative stress (OS) that results from an imbalance between formation and neutralization of pro-oxidants. Initiation of OS is due to free radicals which become stable through electron pairing with biological macromolecules. This causes protein and DNA damage progressively leading to lipid peroxidation. It has been observed that OS has been a major cause for pathogenesis of cancer, atherosclerosis, diabetes, ageing, inflammatory diseases and cardiovascular diseases (Uttara et al., 2009; Halliwell and Gutteridge, 1984). The damage can become more widespread due to weakened cellular antioxidant defence systems. All biological systems have antioxidant defence mechanisms that protects against oxidative damages and repairs enzymes to replace affected molecules. Dietary intake of antioxidant is important to stabilise the natural antioxidant defence mechanism.

Antioxidants are substances that prevent damage to cells...
caused by free radicals by supplying electron to these free radicals. Damage to the adjacent cells is thus prevented by the adjacent cells is thus prevented by the molecules. They additionally change free radicals into waste by-products that eventually get eliminated from the body. Moreover, regular intake of fruits and vegetables is understood to lower the chance of many diseases, like cancer, vessel diseases and stroke caused by OS and such health benefits are primarily for the phytochemicals, such as poly-phenols, carotenoids and flavonoids (Bhatt et al., 2005).

_Sapindus trifoliatus_ belongs to the family Sapindaceae and is widely spread in India, Sri Lanka. The bark of the plant is astringent, sweet, refrigerant, carminative, diuretic, digestive, antihelminthic, febrifuge, respiratory diseases (Muruganandan et al., 2001), diarrhoea (Caceres et al., 1993) and antibacterial. The fruits and seeds are used to treat diabetes (Chakraborty et al., 1986), dysentery (Chopra et al., 1958), allergic disorders (Kim et al., 1958), urethrorrhoea and ringworm infection. The leaves have been extensively used to treat diabetes, constipation (Kim et al., 1998), leucorrhoea, stomachalgie, fever, gastropathy, strangury and dermopathy (Warrier et al., 1996) and to inhibit blood discharges in the faeces (Bhandary et al., 1995).

_S. trifoliatus_ (ST) have been selected since the information of medicinal values on it is still lacking in the literature. Therefore, the research was carried out to enrich the information of the medicinal property of _S. trifoliatus_ in terms of antioxidant and anticancer as well as its polyphenolic and flavonoid contents in the literature.

**METHODOLOGY**

**Collection of plant materials**

Leaves of ST were collected from Utkal University Campus, Bhubaneswar, Odisha, India in June 2012 and were identified by an expert taxonomist at Botany Department, Utkal University where a voucher specimen was deposited (Voucher specimen No:123-ST/BOT). Plant materials were then washed with fresh water to remove dirty materials and were shade dried for several days. They were sun dried sometimes. The dried materials were pulverised into coarse powder by grinding machine, and were stored at room temperature for future use.

**Preparation of extract**

The powdered plant materials of about 700 g were taken in an extraction bottle of 2.5 L capacity and they were soaked with methanol (1 L x 3 times) with timely stirring for 7 days. The extracts were filtered through cotton and filter paper. It was then concentrated with a rotary evaporator under reduced pressure at 45°C to afford 50 g crude extract. The extract was then fractionated successively to obtain petroleum ether fraction (PEF, 15.54 g), chloroform fraction (CHF, 11 g), ethyl acetate fraction (EAF, 8.68 g) and aqueous fraction (AOF, 14.50 g).

**Chemicals**

Catechin (CA), ferrous ammonium sulphate, butylated hydroxyl toluene (BHT), gallic acid (GA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin (QU), DMSO, EDTA, thiobarbituric acid (TBA), acetyl acetone and FeCl3, potassium ferricyanide, potassium acetate, phosphate buffer, ascorbic acid (AA), AlCl3, trichloro acetic acid (TCA), sodium phosphate, ammonium molybdate, and tannic acid were purchased from Sigma-Aldrich Chemical Co., (Bangalore); vanillin was procured from BDH; Folin-Ciocalteu’s phenol reagent and sodium carbonate were also obtained from Sigma-Aldrich Chemical Co., (Bangalore).

**Estimation of total phenolics**

The extracts were taken for estimation of total phenolic contents by the modified Folin-Ciocalteu method (Wolfie et al., 2003). An aliquot of the extracts/standard was mixed with 2 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 2 ml (75 g/L) of sodium carbonate. The tubes were vortexed for 15 s, and allowed to stand for 20 min at 25°C for colour development. Absorbance was then measured at 760 nm UV-spectrophotometer (Shimadzu, USA). Samples of extracts standard were evaluated at a final concentration of 0.1 mg/ml. Total phenolic contents were expressed in terms of gallic acid equivalent (GAE; standard curve equation: y = 0.011x+0.066, \( R^2 = 0.997 \)), mg of GA/g of dry extract.

**Total flavonoids estimation**

From the leaves, total flavonoids were estimated using the following method (Ordonez et al., 2006). To 0.5 ml of samples/standard, 1.5 ml of methanol, 100 μl of 10% AlCl3, 100 μl of 1 M CH3COOK solution and 2.8 ml of distilled water was added. After 1 h 30 min of incubation at room temperature (RT), the absorbance was recorded at 420 nm. The samples and the standard was evaluated at a final concentration of 0.1 mg/ml. Total flavonoid contents were expressed in terms of catechin equivalent (CAE; standard curve equation: \( y = 0.003x+0.022, R^2 = 0.996 \) ), mg of CA/g of dry extract.

**Estimation of total flavonols**

Total flavonols in the leaf extracts were estimated using the following method (Kumaran and Karunakaran, 2007). To 2.0 ml of sample/standard, 2.0 ml of 2% AlCl3 ethanol and 3.0 ml (50 g/L) sodium acetate solutions were added. The absorption at 450 nm was read after 2.5 h at 20°C. Extractives/standard were evaluated at a final concentration of 0.1 mg/ml. Total content of flavonols was expressed in terms of quercetin equivalent, QUE; standard curve equation:

\[ y = 0.2055x+0.0069, \quad R^2 = 0.999 \text{ mg of QU/g of dry extract.} \]

**Estimation of total proanthocyanidins**

From the leaf extract, the content of proanthocyanidins was estimated based on the following method (Sun et al., 1998). From extracts, a volume of 0.5 ml of 0.1 mg/ml standard solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm and samples; standard was evaluated at a final concentration of 0.1 mg/ml. Total content of proanthocyanidins was expressed in terms of catechin equivalent,
CAE; standard curve equation:

\[ y = 0.567x - 0.024, \quad R^2 = 0.982 \text{ mg of CA/g of dry extract.} \]

Determination of antioxidant activity

Estimation of total antioxidant capacity

Total antioxidant capacity (TAC) of samples and standard was determined by the following method (Prieto et al., 1999). Briefly, 0.3 ml of extracts in methanol (1 mg/ml) was added to 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The solutions formed were incubated at 95°C for 90 min. After cooling to room temperature, the absorbance was recorded at 695 nm. The results were evaluated through the standard curve of AA obtained by the same procedure. Increased absorbance of the reaction mixture indicated increase in total antioxidant capacity.

Determination of ferrous ions (Fe²⁺) reducing antioxidant capacity

Different concentrations were taken for the ferrous reducing power of samples; standard was evaluated by the method of Oyaizu (1986). 0.25 ml samples in standard solution at different concentrations, 0.625 ml of potassium buffer (0.2 M) and 0.625 ml of 1% potassium ferricyanide [K₃Fe(CN)₆] solution were added into the test tubes. The mixture was incubated at 50°C for 20 min. After incubation, 0.625 ml of TCA (10%) was added to terminate the reaction and centrifuged at 3000 rpm for 10 min. The upper portion of the solution (1.8 ml) was mixed with 1.8 ml distilled water, and 0.36 ml FeCl₃ solution (0.1%) was added and the absorbance was measured at 700 nm against an appropriate blank solution. Ascorbic acid at various concentrations (10 to 100 μg/ml) was used as standard. Increased absorbance of the reaction mixture indicated increased reducing power.

DPPH free radical scavenging assay

Free radical scavenging activity was determined by DPPH radical scavenging assay (Choi et al., 2000). A solution of 0.1 mM DPPH in methanol was prepared and 2.5 ml of this solution was mixed with 1.5 ml of extracts in methanol at different concentrations. The mixture was mixed thoroughly and left in the dark at room temperature for 30 min. At 517 nm, the absorbance of the mixture was measured spectrophotometrically. BHT was used as reference standard. DPPH radical scavenging activity was calculated by the following equation:

\[ \text{DPPH radical scavenging activity} (\%) = \left( \frac{A_0 - A_t}{A_0} \right) \times 100 \]

where A₀ is the absorbance of the control and Aₜ is the absorbance of the extractives/standard. Then percentage of inhibition was plotted against concentration, and from the graph, IC₅₀ was calculated.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extractives/standard was determined by the following method (Klein et al., 1981) with some changes. 0.5 ml of extractives/standard at various concentrations was taken in different test tubes. 1 ml of Fe-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of 0.018% EDTA solution, 1 ml of 0.85% DMSO solution and 0.5 ml of 22% AA were added into each of the test tubes. The test tubes were closed tightly and warmed for 15 min at 85°C into the water bath. After incubation, the test tubes were opened and 0.5 ml ice cold TCA (17.5%) was added to each of the test tubes immediately. 3 ml of reagent (7.5 g of ammonium acetate, 300 μl glacial acetic acid and 200 μl 2,4-pentanediol was mixed and adjusted to 100 ml) was added into all the test tubes and incubated at room temperature for 15 min. Absorbance was taken at 412 nm. Percentage hydroxyl radical scavenging activity was calculated by the following equation:

\[ \text{Hydroxyl radical scavenging activity} (\%) = \left( \frac{A_0 - A_t}{A_0} \right) \times 100 \]

where A₀ is the absorbance of the control and Aₜ is the absorbance of the extractives/standard. Then percentage of inhibition was plotted against concentration and IC₅₀ was calculated.

Lipid peroxidation inhibition assay

The lipid peroxidation inhibition assay was determined according to the method described by Liu et al. (2000) with a slight modification. Excised rat liver was homogenized in buffer and then centrifuged to obtain liposome. 0.5 ml of supernatant, 100 μl of 10 mM FeSO₄, 100 μl of 0.1 mM AA and 0.3 ml of extractives/standard at different concentrations were mixed to make the final volume of 1 ml. The reaction mixture was incubated at 37°C for 20 min. One millilitre of 28% TCA and 1.5 ml of 1% TBA was added immediately after heating. Reaction mixture was again heated for 15 min at 100°C and cooled at room temperature. After cooling, the absorbance was taken at 532 nm. Inhibition of lipid peroxidation was calculated by the following equation:

\[ \text{Lipid peroxidation inhibition} (\%) = \left( \frac{A_0 - A_t}{A_0} \right) \times 100 \]

where A₀ is the absorbance of the control, and Aₜ is the absorbance of the extractives/standard. Then percentage of inhibition was plotted against concentration and IC₅₀ was calculated from the graph.

Determination of anticancer activity

Cell growth inhibition

Cell growth inhibition was carried out by the method described by Sur and Ganguly (1994). Protocol used in this study for the use of mice as animal model for cancer research was approved by the Institutional Animal Ethical Committee, UDPS, Utkal University of CPCSEA (Government of India, Regn. No-990/c/CPCSEA/05).

In this research study, four groups of mice (6 in each group) were used on Day 1. 14×10² Ehrlich’s Ascites cells (EAC)/mouse as inoculated into each group of mice. After 24 h of EAC inoculation treatment was started which continued for 5 days. Test compound (25 and 50 mg/kg) was given to Groups 1 and 2, respectively per day per mouse. In each case, the volume of the test solutions injected intraperitoneally (i.p.) was 0.1 ml/day per mouse. Group 3 received standard bleomycin (0.3 mg/kg, i.p) and were considered as positive control. Vehicle (normal saline) was given to Group 4 and was considered as negative or untreated control. On the 6th day after collection of tumour cells by repeated i.p. wash with 0.9% saline, the mice were sacrificed. Viable tumour cells per mouse of the treated group were compared with those of the control. The cell growth inhibition was calculated using the following formula:

\[ \text{Cell growth inhibition} (\%) = \left( 1 - \frac{T_w}{C_w} \right) \times 100 \]
Figure 1. Determination of (A) total antioxidant capacity and (B) ferrous reducing antioxidant capacity of CME and its various fractions (PEF, CHF, EAF and AQF). Data expressed as mean ± standard deviation (SD; n=3, p < 0.05) for all tested doses.

RESULTS

TAC and ferrous reducing antioxidant capacity estimation

The TAC of CME and its four fractions of leaves of ST are as shown in Figure 1A. CME of leaves of ST showed higher antioxidant activity as compared to the reference standard CA at all the concentrations. The absorbance of
CME, PEF, CHF, EAF, AQF, and standard CA were 1.90, 2.21, 0.96, 2.65, 1.49 and 1.35, respectively at 324 μg/ml. The TAC of EAF was significantly higher (p < 0.01) than standard CA. The extractives were found to increase the total antioxidant activity with the increasing concentration of the extracts.

The ferrous reducing antioxidant capacity of CME and its four fractions are as shown in Figure 1B. At 160 μg/ml, the absorbance of CME, PEF, CHF, EAF, AQF, and standard AA were 2.75, 2.53, 2.21, 2.98, 2.91 and 2.32, respectively. A higher absorbance indicates a higher reducing power, hence CME, EAF and AQF showed higher reducing activity than standard AA. The ferrous reducing capacity of EAF was significantly higher (p < 0.01) than standard AA. CHF and PEF had mild to moderate iron reducing capacity. It was observed that with increasing concentration of the extracts reducing activity also increased.

**DPPH radical scavenging activity**

Figure 2A showed free radical scavenging activity of the CME and its four fractions. At a concentration of 25 μg/ml, the scavenging activity of the CME, EAF and AQF were 95.93, 95.67 and 93.41%, respectively, while at the same concentration, the activity of BHT was 88.53%. Thus, CME, EAF and AQF exhibited significant free radical scavenging activity (Figure 2A). The scavenging activity of the PEF and CHF was in moderate level when
compared with BHT (Figure 2A). The IC\textsubscript{50} of CME, PEF, CHF, EAF and AQF were 9.92, 63.2, 23.5, 95.93 and 10.0 μg/ml, respectively. The IC\textsubscript{50} of BHT was 9.83 μg/ml, which was almost double than the IC\textsubscript{50} of EAF (4.55 μg/ml). The inhibitory activity of different extractives and BHT were in the following order: EAF > BHT > CME > AQF > CHF > PEF. Our results revealed that the EAF had higher scavenging activity than that of other extractives as compared to BHT and CME and AQF had similar activity with BHT.

**Hydroxyl radical scavenging activity**

At a concentration of 150 μg/ml, the scavenging activity of CME and its four fractions PEF, CHF, EAF and AQF reached 88.29, 51.03, 57.85, 87.15 and 63.95%, respectively; while at the same concentration, the activity of AA was 84.25% (Figure 2B). The IC\textsubscript{50} of CME, PEF, CHF, EAF, AQF and AA were 50.25, 145.29, 133.45, 43.33, 124.65 and 32.15 μg/ml, respectively. The result demonstrates that CME and EAF significantly scavenged hydroxyl radicals when compared with standard AA.

**Lipid peroxidation inhibition assay**

The lipid peroxides scavenging activity of CME of leaves of ST was investigated and compared with standard CA. At a concentration of 150 μg/ml, the scavenging activity of CME and its fractions, PEF, CHF, EAF and AQF were 79.25, 56.35, 54.98, 81.65 and 66.12%, respectively; whereas the activity of CA was 80.54% (Figure 3A). The EAF exhibited higher activity than other extractives, even though higher than standard CA. The IC\textsubscript{50} of CME, PEF, CHF, EAF and AQF were 72.52, 135.34, 135.25, 70.15 and 100.25 μg/ml, respectively; on the other hand, the IC\textsubscript{50} of CA was 58.25 (Figure 3B). Significant correlations (p < 0.001) were observed between percentage of lipid peroxidation inhibition and hydroxyl radical scavenging (Figure 3).

**Total polyphenol contents**

**Tumor cell growth inhibition**

Since EAF showed the strongest antioxidant activity in all antioxidant tests, it was chosen for in vivo tumor cell growth inhibition at various doses (25 and 50.0 mg/kg, i.p). Maximum cell growth inhibition (67.35%, p < 0.01) was found after treatment with EAF at dose 50.0 mg/kg (i.p) on day six of tumor inoculation. On the other hand, standard bleomycin at dose 0.3 mg/kg i.p inhibited the tumor cell growth by 83.81% (Table 2). This result implies that the EAF had moderate anticancer activity, and the plant might therefore be considered as an effective source of active chemopreventive agents.

**DISCUSSION**

**Total antioxidant capacity**

Total antioxidant potentials of leaves of *S. trifoliatus* extracts were estimated from their ability to reduce the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The antioxidant potential of the extractives was in the range of 0.95±0.003 to 2.65±0.004 μm green phosphate/Mo (V) (Figure 1A). All the fractions showed a good total antioxidant activity, which was concentration-dependent. The antioxidant potential of EAF was significantly higher (p < 0.05) than standard antioxidant which may be due to its chemical composition and phenolic content.

**Ferrous reducing antioxidant capacity**

Reducing power is also widely used in evaluating antioxidant activity of plant polyphenols and reductones that show antioxidant action by breaking the free radical chains by donating a hydrogen atom. The presence of reductants in the antioxidant sample causes the reduction of the Fe\textsuperscript{3+}/ferricyanide complex to the Fe\textsuperscript{2+}/ferrous form. The reducing power of the sample can be observed by measuring the formation of Perl's Prussian blue at 700 nm. The reducing ability of the extractives was in the range of 1.32±0.004 to 2.96±0.002 μm Fe (II)/g. The EAF exhibited strong reducing power and was higher than other fractions, even significantly higher (p < 0.05) than AA as shown in Figure 1B.

**DPPH radical scavenging activity**

DPPH radicals antioxidant activity is thought to be due to their hydrogen donating potential (Klein et al., 1981). In order to prevent the deleterious role of free radical in different diseases including cancer, radical scavenging activities are necessary. Relatively short time is required for this analysis which makes this method popular among the researchers. DPPH radical scavenging activity of all the fractions from *S. trifoliatus* increased with increase in fraction concentration (Figure 2A). The IC\textsubscript{50} of EAF was significantly higher (p < 0.01) than that of other fractions and BHT with the order of EAF > BHT > CME > AQF > CHF > PEF. It has been found that mainly phenolics and flavonoids have a vital role in reducing the DPPH radicals by their hydrogen donating ability (Nariya et al., 2013). The results obtained in this study reveal that all the fractions from *S. trifoliatus* leaves are free radical scavengers.
and are able to react with the DPPH radical, which might be responsible for the antioxidant potential.

**Hydroxyl radical scavenging activity**

Free radicals mutagenic capacity is due to the direct interactions of hydroxyl radicals with DNA and therefore playing an important role in cancer formation (Rahman, 2007). Hydroxyl radicals can be generated by biochemical reaction. The results reveal that EAF of the leaves of *S. trifoliatus* had appreciable hydroxyl radical scavenging activity when compared with standard antioxidant BHT (Figure 2B) and could be used as anticancer agent by inhibiting the interaction of hydroxyl radical with DNA. Inhibition of lipid peroxidation may result due to quenching of the hydroxyl radicals by the extract.

**Lipid peroxidation inhibition assay**

The reactive oxygen species (ROS) induced membrane damage by peroxidising lipid moiety, especially the polyunsaturated fatty acids with a chain reaction known as lipid peroxidation (Mylonas and Kouretas, 1999). Lipid peroxidation has been reported to be elevated in the cancer (Cai et al., 2012). In this study, lipid peroxidation of mouse liver homogenates was induced by ferric ion plus ascorbic acid. The CME of the leaves of *S. trifoliatus* and its four fractions, especially EAF had appreciable lipid peroxidation inhibition activity (Figure 3). The ST
extracts can prevent the cell abnormalities caused by cancer through breaking down of chain reactions responsible for lipid peroxidation. Inhibition of lipid peroxidation by the EAF fraction was significantly correlated with hydroxyl radical scavenging (Figure 3). This result reveals that the extractives differentially inhibit lipid peroxidation by virtue of their varying degrees of free radical quenching potential. Thus, ST is a good source for antioxidant thereby can be used as anticancer agent.

Table 1. Total polyphenols contents in the CME and its four fractions: PEF, CHF, EAF and AQF.

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>CME</th>
<th>PEF</th>
<th>CHF</th>
<th>EAF</th>
<th>AQF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics*</td>
<td>301.63 ± 4.36³</td>
<td>17.56 ± 1.41</td>
<td>118.13 ± 1.61</td>
<td>526.22 ± 1.55</td>
<td>266.88 ± 2.78</td>
</tr>
<tr>
<td>Flavonols**</td>
<td>119.38 ± 1.24</td>
<td>185.48 ± 1.19</td>
<td>149.01 ± 2.78</td>
<td>220.38 ± 1.26</td>
<td>132.54 ± 1.77</td>
</tr>
<tr>
<td>Flavonoids***</td>
<td>219.88 ± 15.17</td>
<td>152.13 ± 6.34</td>
<td>128.21 ± 9.38</td>
<td>612.75 ± 5.37</td>
<td>185.71 ± 11.35</td>
</tr>
</tbody>
</table>

Each value is the average of 3 analyses ± standard deviation (SD). *, ** and *** expressed in terms of GAE, QUE and CAE, respectively.

Table 2. Effect of EAF on EAC cell growth inhibition in mice (in vivo).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg/day; i.p)</th>
<th>No. of EAC cells in mouse×10⁷ on day 6 after tumour cell inoculation</th>
<th>Cell growth inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (EAC cell bearing mice)</td>
<td>-</td>
<td>5.12 ± 0.23</td>
<td>-</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>0.3 mg/kg</td>
<td>0.73 ± 0.03**</td>
<td>85.92</td>
</tr>
<tr>
<td>EAF</td>
<td>25 mg/kg</td>
<td>2.2 ± 0.23*</td>
<td>50.45</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg</td>
<td>1.13 ± 0.12**</td>
<td>65.14</td>
</tr>
</tbody>
</table>

n=6, results were shown as mean ± standard error of mean (SEM), *p < 0.05, **p < 0.01.

Total phenolic, flavonoids, flavonols and proanthocyanidin contents

A number of polyphenols are identified as potential anti-tumor agents that could be used to combat biologically aggressive cancers, including metastasizing cancers, through the targeting of specific kinases (Lamoral-Theys et al., 2010). Secondary metabolites like flavonoids have good antioxidant potential which have also been shown to possess antimutagenic and antimalignant effect (Lamoral-Theys et al., 2010). Furthermore, flavonoids have a chemopreventive role in cancer through their effect on signal transduction in cell proliferation and angiogenesis (Ramos, 2007). Dietary flavonols and proanthocyanidins in particular offer significant cardiovascular health benefits (Ramos, 2007). Results obtained in the present study revealed that the level of these phenolic compounds in leaves of ST were significant (Table 1). Our findings strongly suggest that the phenolics are important components of ST pharmacological effects like anticancer activity and antioxidant activity could be due to the presence of these metabolites.

Conclusion

The present study shows that as compared to the other fractions, the ethyl acetate fraction of *S. trifoliatus* possessed
the highest phenolic content and also exhibited strong antioxidant with moderate anticancer activities. Thus, it can be concluded that the S. trifoliatus extract can be used as natural antioxidant as well as an anticancer agent. Further analysis and characterization of the phenolic compounds may help in discovering the chemical moieties responsible for the antioxidant and anticancer activities from the ethyl acetate fraction of S. trifoliatus.

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ABBREVIATIONS

AA, Ascorbic acid; AQF, aqueous fraction; CAE, catechin equivalent; CHF, chloroform fraction; CME, crude methanolic extract; DPPH, 1,1-diphenyl-2-picrylhydrazine; EAC, Ehrlich's Ascites cells; EAF, ethyl acetate fraction; GA, gallic acid; GAE, gallic acid equivalent; OS, oxidative stress; PEF, petroleum ether fraction; QE, quercetin equivalent; ROS, reactive oxygen species; RT, room temperature; ST, Sapindus trifoliatus; TAC, total antioxidant capacity; TCA, trichloro acetic acid.

Conflict of Interest

The authors declare that there is no conflict of interest.

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