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Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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ARTICLES

Research Articles

- Antispasmodic potential of leaves, barks and fruits of *Zanthoxylum armatum* DC** 685
Barakat Barq Barkatullah, Muahmmad Ibrar, Niaz Ali,
Naveed Muhammad, Rehmanullah
- Comparison of hydroxyethyl starch and succinylated gelatin in adaver renal graft function in kidney-transplant recipients** 694
Bin Liu, Meihua Piao, Chunyan Zhou, Yaowen Fu, Chunsheng Feng
- Evaluation of the phenolic and flavonoid contents and radical scavenging activity of three southern African medicinal plants** 703
Steenkamp V., Nkwane O., van Tonder J., Dinsmore A., Gulumian M
- The clinical characteristics of anti-tuberculosis drug induced liver injury in 2457 hospitalized patients with tuberculosis in China** 710
Huiru An, Xueqiong Wu, Zhongyuan Wang, Jing Xu, Shaohua Zheng, Kun Wang
- Incidence, type and causes of dispensing errors: A study from the community pharmacy** 715
Alina de las Mercedes Martínez Sánchez

ARTICLES

Research Articles

- Study on mock strawberry extracts *in-vitro* anti-tumor activity** 721
Zhang Lili, Zhang Lan, Wang Xiangming, Gu Jinxia, Wang Xiaoyun,
Li Xueqi, Xia Hongyuan
- Antibacterial activities, chemical constitutes and acute toxicity of Egyptian
Origanum majorana L., *Peganum harmala* L. and *Salvia
officinalis* L. essential oils** 725
Samy A. Selim, Mohamed H. Abdel Aziz, Mona S. Mashait,
Mona F. Warrad

Full Length Research Paper

Antispasmodic potential of leaves, barks and fruits of *Zanthoxylum armatum* DC

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Zanthoxylum armatum is a well known food product used as antispasmodic in traditional medicine system. In the present research work, the phytochemical composition of the leaves, barks and fruits was carried out showing that all parts are good source of alkaloids, saponin, tannins and flavonoids. The ethanolic and n-hexane extracts were tested for their antispasmodic potential using rabbit ileum tissues. The ethanolic and n-hexane extract of the leaves (ZLE and ZLH), barks (ZBE and ZBH) and fruits (ZFE and ZFH), demonstrated a dose dependant antispasmodic effect. In most of the tested extracts, the action of ethanolic extract was found more pronounced than the other. The most significant antispasmodic effect was observed with fruit extracts. In conclusion, the current study strongly supports the folkloric uses of the plant as antispasmodic.

Key words: *Zanthoxylum armatum*, rabbit ileum, phytochemical studies, folkloric, antispasmodic.

INTRODUCTION

Developing countries have been facing morbidity and mortality of childhood due to diarrhea and other gastrointestinal problems. An estimated frequently 10 million deaths per year in children under age of 5 has been reported (Carlos and Sanieel, 1990). In these countries, people are still relying on the herbal drugs for control of diarrhea despite of enormous development of all anti-diarrheal medicines in the world (Ojewole, 2004; Agunu et al., 2005). The world health organization (WHO) highly appreciated the conventional medical practices for treatment and precautionary measure of diarrheal diseases (Atta and Mouneir, 2004).

Zanthoxylum armatum is a small xerophytic tree or shrub, with leaflet blades usually with prickles. The leaves are compound, imparipinnate with 3 to 7 foliolate and pellucid-punctate. The petiole and rachis are winged. The leaflets are sessile, elliptic to ovate-lanceolate with

crenate or entire margins. The flowers are born axillary, minute and polygamous. Calyx is 6 to 8 acute lobed. Petals are absent. Male flowers are always with 6 to 8 stamens with rudimentary ovary, while female flowers are with 1 to 3 carpels. Ovary is 1 to 3 locular. Fruit is small drupes with red color, splitting into two when ripe. The seeds are rounded and shining black (Hassan-Ud-Din and Ghazanfar . *Z. armatum* prefers semi shady or no shade for growth. It grows wild in foothills starting from about 800 to 1500 m in Malakand, Swat, Dir, Hazara, Buner, Muree hills and Rawalpindi (Shinwari et al., 2006). In Pakistan, it is known as Dambrary, Tamur (Urdu) and Dambara (Pashtu). Its fruits and seeds are edible and used as potherb species. The plant is used for pneumonia and tick infestation (Sindhu et al., 2010). Young shoots are used as toothbrush and useful for curing gum diseases. The fruit is used for toothache,

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dyspepsia, as a carminative and stomachache. The seeds are used as condiment and flavoring agent. The wood is used to make walking sticks (Arshad and Ahmad, 2004; Abbasi et al., 2010). Powdered fruit, mixed with *Mentha* species and table salt is eaten with boiled egg for chest infection and other digestive problems (Islam et al., 2009). Recently, the leaves and fruits of this plant were tested for various pharmacological activities including antipyretic action (Barkatullah et al., 2011).

In this study, ethanolic and n-hexane extracts of the leaves, barks and fruits of *Z. armatum* were evaluated on isolated rabbit jejunum preparations, to rationalize the ethnopharmacological use of this important medicinal plant.

MATERIALS AND METHODS

Drugs and other chemicals

Acetylcholine (BDH Chemicals, Poole, England), potassium chloride (KCl; E. Merck Germany), Tyrode's solution (Prepared from its constituents with their respective concentrations (mM): NaCl, 136.9; KCl, 2.68; MgCl₂, 1.05; NaH₂PO₄, 0.42; NaHCO₃, 11.90; CaCl₂, 1.8; and glucose, 5.55; dissolved in 1 L distilled water).

Animals

Local breed rabbits of either sex with weights ranging from 1.0 to 1.4 kg were used. The animals were kept for 14 days before starting the experiments at the "Animal House of the Department of Pharmacy, University of Malakand" under standard conditions mentioned in the "Animals Bye-Laws 2008 of the University of Malakand (Scientific Procedures Issue- 1)", and were fed on standard diet and tap water. The animals were kept in fasting condition 24 h prior to the start of experiments with free excess to water.

Qualitative chemical identification tests

Various phytochemical tests were performed for detection of various constituents preliminary, using well established procedures (Trease and Evans, 1989; Muhammad and Saeed, 2011). In case of quantitative phytochemical study, alkaloids, saponin, tannins and flavonoid were determined following the published protocols (Harborne, 1998; Huang et al., 2010; Muhammad and Saeed, 2011; Barkatullah et al., 2012).

Rabbit's jejunum preparations for antispasmodic activity

Slaughtered animals were dissected to open abdomen and jejunum portion(s), extracted and kept in freshly prepared Tyrode's solution, aerated with carbogen gas (5% carbon dioxide and oxygen mixture) to keep them alive and ready for use. Quiescent sub-maximal doses of acetylcholine (0.3 μ M) to the tissues were used when needed for keeping the tissue viable and alive. About 1.5 cm length tissue was mounted in 10 ml tissue bath containing Tyrode's solution and was stabilized for 25 to 30 min. All the processes were carried out at 37 \pm 1°C with constant aeration and kept under 1 g pressure. On attaining reproducible response, test samples at the doses of 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 5.0, and 10.0 mg/ml were

applied to the bath solution (Gilani et al., 2005b; Ali and Shah, 2010). The processes were repeated thrice (n=3) and fall in spontaneous activity was observed to be change of the sample tested. For the determination of possible mode of action, the tissue was pretreated with high concentration of KCl (80 mM in final bath solution). KCl cause depolarization and the tissue was kept in a position of sustained contraction. The extract was then applied in cumulative manner to obtain a dose dependent curve and relaxation. Intestinal responses data were recorded using Force Transducer (Model No: MLT 0210/A Pan Lab S.I.) attached with Power lab (Model No: 4/25 T) AD Instruments, Australia. Data was recorded at range of 20 mV, low pass at 5 Hz \times 10 gain using input 1, rate 40 s⁻¹ (Ali and Shah, 2010). Results were expressed as percentage of KCl induced contraction.

Statistics and interpretation

Chart 5 (AD Instruments) was used to interpret the graph tracings. Student "t" test was used at 95% confidence interval (CI). 'P' values less or equal to 0.05 was considered as statistically significant.

RESULTS

Qualitative phytochemical screening

Qualitative phytochemical screening was carried out using different extracts of the leaf, stem bark and fruit of *Z. armatum*. The detail of this study is shown in Tables 1, 2 and 3, respectively.

Quantitative phytochemical screening

Bioactive constituents like alkaloids, sterol, saponins, tannins, phenols and flavonoids were quantitatively evaluated in leaf, bark and fruit of *Z. armatum*. The results revealed the presence of bioactive constituents in leaves comprising of alkaloids (15.60 \pm 0.10 mg/g), sterols (71.60 \pm 0.10 mg/g), saponins (21.57 \pm 0.12 mg/g), tannins (34.43 \pm 0.21 mg/g), phenols (11.66 \pm 0.33 mg/g), and flavonoids (13.68 \pm 0.66 mg/g). The bark contained alkaloids (19.60 \pm 0.10 mg/g), sterols (33.83 \pm 0.29 mg/g), saponins (14.78 \pm 0.10 mg/g), tannins (28.62 \pm 0.13 mg/g), phenols (16.48 \pm 1.33 mg/g), and flavonoids (18.33 \pm 1.22 mg/g), while the fruits contained alkaloids (25.07 \pm 0.21 mg/g), sterols (164.92 \pm 0.14 mg/g), saponins (28.60 \pm 0.10mg/g), tannins (35.5 \pm 0.5 mg/g), phenols (21.68 \pm 0.44 mg/g) and flavonoids (22.8 \pm 1.33 mg/g) (Table 5). Comparative statistics of these bioactive constituents in the leaves, bark and fruit of *Z. armatum* are as shown in Figure 3.

Antispasmodic activity

Antispasmodic potential of all samples from *Z. armatum* is shown in Figures 1 and 2. The effect of ethanolic extract of *Z. armatum* leaves (ZLE) was found to start from 0.3 mg/ml and reached to maximum at a dose of 10 mg/ml.

Table 1. Preliminary phytochemical screening of *Zanthoxylum armatum* leaves.

Constituent	Test name	Ethanollic extract	Hexane extract
Carbohydrates	Fehling's test	+	-
	Molisch's test	+	-
	Benedict's test	+	-
Protein	Ninhydrin test	-	-
	Millon's test	-	-
Alkaloids	Wagner's test	+	+
	Mayer's test	+	+
	Hager's test	+	+
Phytosterol and Triterpenoids	Salkowski's test	+	+
	Liebermann-Burchard test	+	+
Phenol	Ferric chloride test	+	-
Flavonoids	Lead acetate test	+	+
	Alkali test	+	+
	Shinoda's test	+	+
Tannins	Gelatin test	+	+
	Ferric chloride test	+	+
	Alkali test	+	+
Saponins	Frothing test	-	-
Anthocyanins	HCl test	+	-
Glycosides	Killer-Kiliani test	-	-
Fixed oil and fats	Spot test	+	+
	Alkali test	+	+
Volatile oil	Spot test	+	+

Table 2. Preliminary phytochemical screening of *Z. armatum* bark.

Constituents	Test name	Ethanollic extract	Hexane extract
Carbohydrates	Fehling's test	+	+
	Molisch's test	+	+
	Benedict's test	+	-
Protien	Ninhydrin test	-	-
	Millon's test	-	-
Alkaloids	Wagner's test	+	-
	Mayer's test	+	-
	Hager's test	+	-
Phytosterol and Triterpenoids	Salkowski's test	+	+
	Liebermann-Burchard test	+	+
Phenol	Ferric chloride test	+	-
Flavonoids	Lead acetate test	+	-
	Alkali test	+	+
	Shinoda's test	+	+

Table 2. Contd.

Tannins	Gelatin test	+	+
	Ferric chloride test	+	+
	Alkali test	+	+
Saponins	Frothing test	-	-
Anthocyanins	HCl test	+	-
Glycosides	Killer-Kiliani test	-	-
Fixed oil and fats	Spot test	+	+
	Alkali test	+	+
Volatile oil	Spot test	+	+

Table 3. Preliminary phytochemical screening of fruit of *Z. armatum*.

Constituent	Test name	Ethanolic extract	Hexane extract
Carbohydrates	Fehling's	+	+
	Molisch's	+	+
	Benedict's	+	-
Protien	Ninhydrin	+	-
	Millon's	+	-
Alkaloids	Wagner's	+	-
	Mayer's	+	-
	Hager's	+	-
Phytosterol and Triterpenoids	Salkowski's	+	+
	Liebermann-Burchard	+	+
Phenol	Ferric chloride	+	-
	Lead acetate	+	-
Flavonoids	Alkali	+	-
	Shinoda's	+	
		+	
Tannins	Gelatin	+	+
	Ferric chloride	+	-
	Alkali	+	-
Saponins	Frothing	-	-
Anthocyanins	HCl	-	-
Glycosides	Killer-Kiliani	-	-
Fixed oil and fats	Spot	+	+
	Alkali	+	+
Volatile oil	Spot	+	+

n-hexane extract of *Z. armatum* leaves (ZLH) showed relaxant effect in the spontaneous tissue treatment. The ethanolic extract of *Z. armatum* bark (ZBE) showed

significant effects observed from 3 to 10 mg/ml concentration. In the case of n-hexane extract of *Z. armatum* bark (ZBH), the antispasmodic effect was

Table 4. EC₅₀ (half maximal effective concentration values) for Ethanolic and n-hexane extract of leaf, bark and fruit of *Z. armatum*.

Part used	Extract	EC ₅₀ value spontaneous rabbit jejunum	EC ₅₀ value for KCl induced contraction
Leaves	ZLE	0.23	6.81
	ZLH	6.13	4.45
Bark	ZBE	4.00	6.00
	ZBH	5.5	4.5
Fruit	ZFE (without atropine)	1.69	7.49
	ZFE (with atropine)	8.86	3.18
	ZFH	0.74	5.00

Table 5. Quantitative chemical analysis of *Zanthoxylum armatum*.

Extract	Flavonoids	Phenol	Alkaloids	Tannins	Saponin	Sterol
ZLE	13.68±0.66	11.66±0.33	15.60±0.10	34.43±0.21	21.57±0.12	71.60±0.10
ZBE	18.33±1.22	16.48±1.33	19.60±0.10	28.62±0.13	14.78±0.10	33.83±0.29
ZFE	22.8±1.33	21.68±0.44	25.07±0.21	35.5±0.5	28.60±0.10	164.92±0.14

All values are mean ± SEM of three determination and are expressed in mg/g.

observed at concentration of 3 mg/ml, while the most significant effect was observed at 10 mg/ml. The ethanolic extract of *Z. armatum* fruit (ZFE) produced strange effect in both cases (spontaneous and in potassium induced contraction). This extract was just like control up to 1 mg/ml and at higher concentrations, a good right ward shift was produced. The EC₅₀ values (half maximal effective concentration values) for ethanolic and n-hexane extract of leaf, bark and fruit of *Z. armatum* are shown in Table 4.

DISCUSSION

Several active constituents of pharmacological importance may be available in a single plant (Ming et al., 2005). It is suggested that preliminary phytochemical screening is important and useful for isolation of pharmacologically active principles from a single plant (Sugumaran and Vetrichelvan, 2008). The qualitative and quantitative analysis of *Z. armatum* showed that this plant might be a rich source of bioactive constituents for future use in pharmaceutical preparations.

Ethanolic and n-hexane extracts of leaves (ZLE and ZLH), barks (ZBE and ZBH) and fruits (ZFE and ZFH) of *Z. armatum* were evaluated on the isolated rabbit jejunum for possible antispasmodic effect, providing scientific proof for its ethnopharmacological use as an antispasmodic drug. All the samples were tested against spontaneous and KCl induced contracted smooth muscle of the isolated rabbit jejunum.

All samples produced antidiarrheal effect in a dose dependent manner. To determine the possible mode of action, the tissue was pretreated with high concentration

of KCl (80 mM in final bath solution). KCl cause depolarization and keep the tissue in a position of sustained contraction (Farre et al., 1991). The test samples were then applied in cumulative manner to obtain a dose dependant curve and relaxation results were expressed as percentage of KCl induced contraction (Van Rossum, 1963).

The effect of ZLE was dose dependent in both spontaneous and KCl induced contractions. The ZLH inhibited the depolarization of the smooth muscles of the jejunum, caused by high concentration of KCl solution (80 mM) (Ahmad, 2010). The ZBE produced spasmolytic effect in both spontaneous as well as in the KCl induced contraction. In case of ZBH, the relaxation of smooth muscle of potassium induced contraction was more than the spontaneous. To confirm that the spasmolytic effect of ZFE was due to possible calcium channel blockage, another series of experiments were performed, in which a pretreated atropine isolated tissues was treated with KCl (80 mM) that opens the voltage operated calcium channels, releasing extra-cellular calcium into the cytosol creating depolarization of the tissue (Gilani et al., 2005a). ZFE was found to relax the KCl depolarized tissue and was found to be calcium channel blocking agent at a dose range of 0.1 to 5.0 mg/ml (Ahmad, 2010) (Figure 1).

The contraction of smooth muscle of rabbit jejunum is due to increase concentration of the free calcium in cytoplasm, which stimulates the chemical mediators responsible for contraction. This increase in calcium level may be either due to influx via voltage dependent calcium channels or direct release of calcium from endoplasmic reticulum (calcium store). Thus a periodic depolarization is created due to high speed action potential. When there is increase potassium concentration, the contraction of

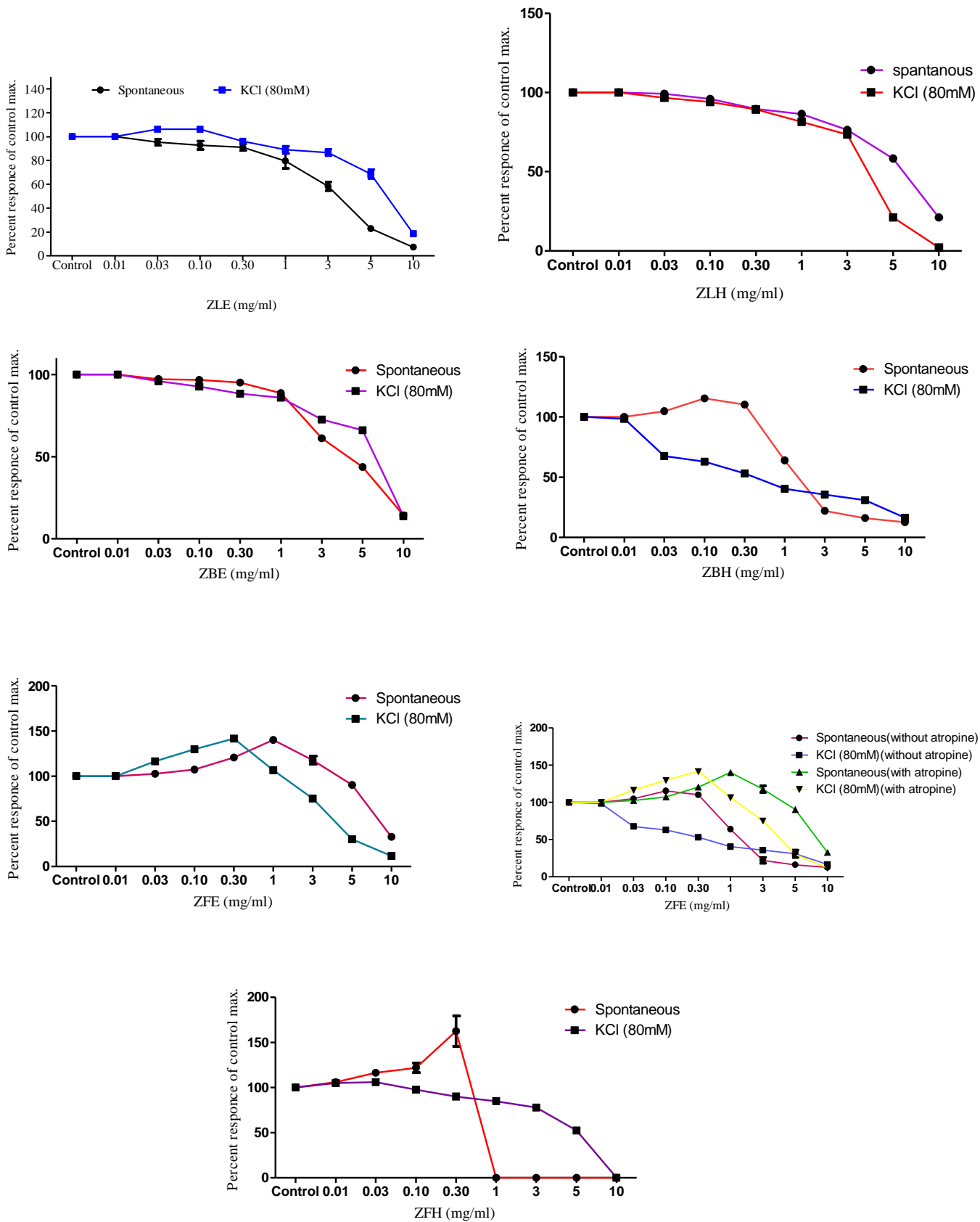


Figure 1. Dose response curve of the ZLE, ZLH, ZBE, ZBH, ZFE, and ZFH on isolated rabbit's jejunum preparations. All values are mean±SEM (n=5).

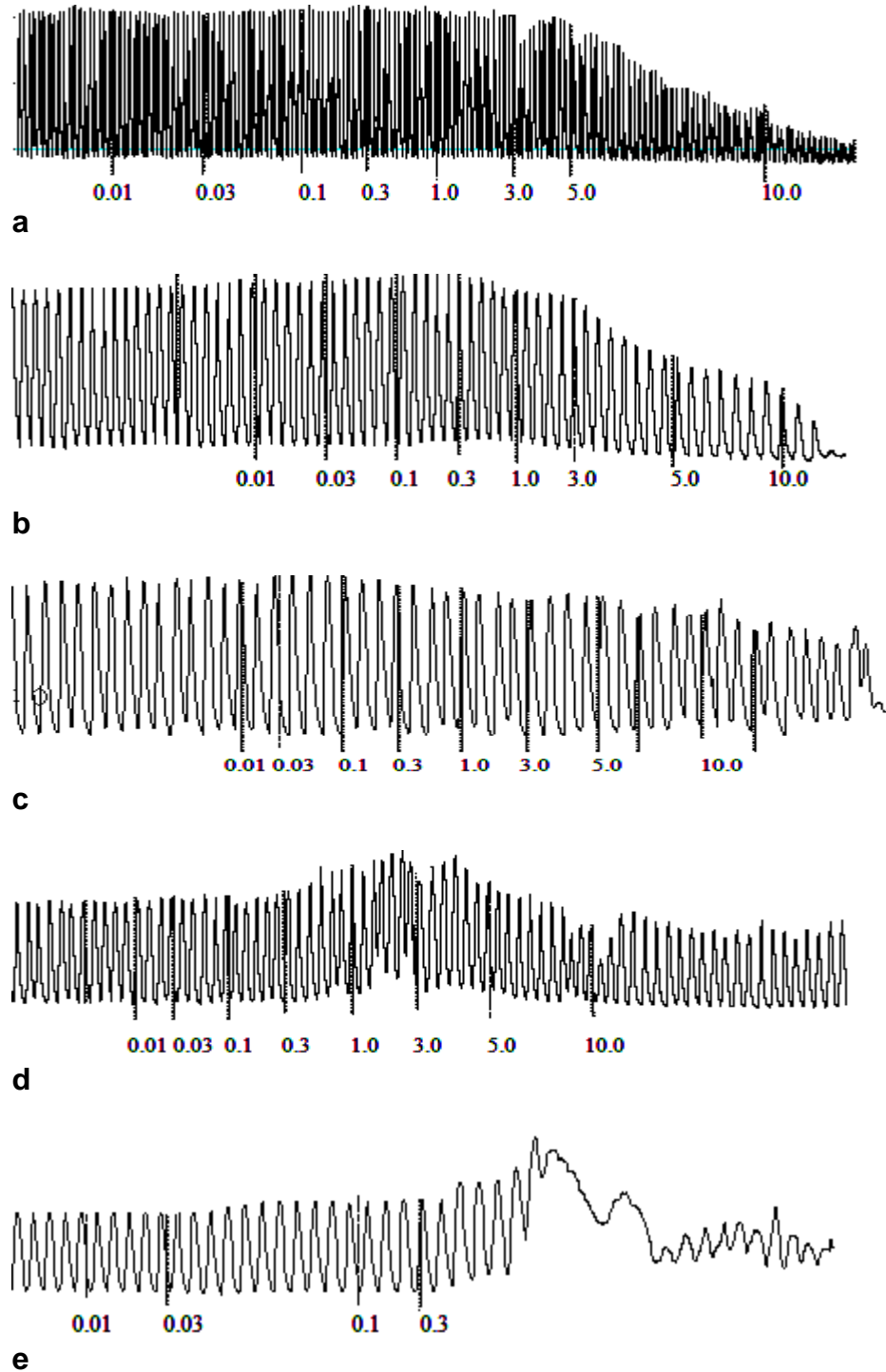


Figure 2. Antispasmodic effect of *Zanthoxylum armatum*. (a) Effect of ZLE (mg/ml) on isolated rabbit jejunum; (b) Effect of ZBE (mg/ml) on isolated rabbit jejunum; (c) Effect of ZBH (mg/ml) on isolated rabbit jejunum; (d) Effect of ZFE (mg/ml) on isolated rabbit jejunum; (e) Effect of ZFH (mg/ml) on isolated rabbit jejunum.

the smooth muscle will increase due to rapid action potential. When the calcium channel is blocked through calcium channel blocker agents, the contracted smooth

muscle will relax (Ali and Shah, 2010). In the present study, the extracts relaxed the contracted muscle, suggesting that the possible mode of action of this plant

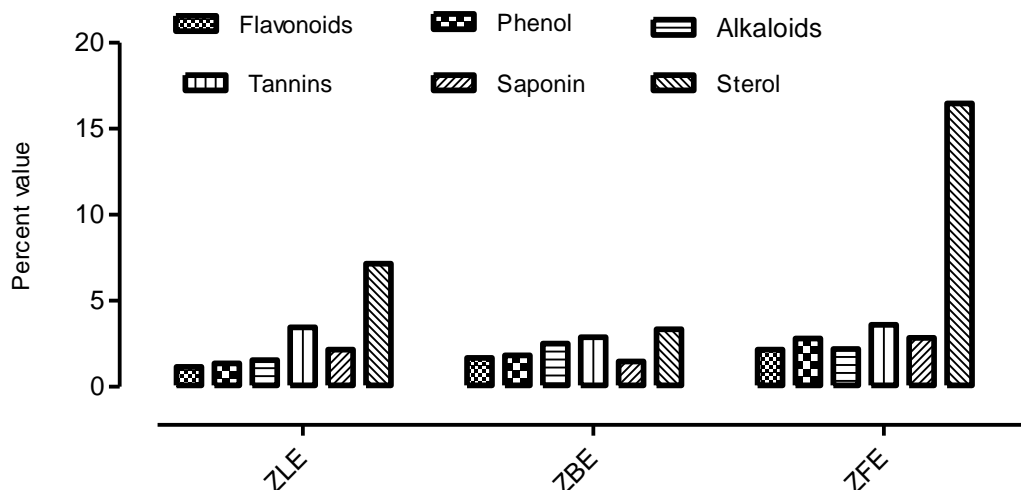


Figure 3. Bars represent percents values of flavonoids, phenols, alkaloids, tannins, saponins and sterols in ethanolic extracts *Zanthoxylum armatum* leaf (ZLE), bark (ZBE) and fruit (ZFE).

is either blocking the release of stored calcium from the sarcoplasmic reticulum or blocking the calcium channel. More or less similar studies have also been carried out by other workers. Ahmad (2010) carried out antispasmodic activities on isolated rabbit jejunum testing the crude methanolic extract of *Tylophora hirsutum*. The methanolic-aqueous extract of the aerial part of *Z. armatum* has been tested for muscles relaxation effect in gut, air passage way and in cardiovascular system (Gilani, 2005a). This study showed that this plant is an important source of active constituents both qualitatively and quantitatively. Further various experiments on this plant demonstrated that this plant has strong antispasmodic potential. Detail studies are required to explore this plant phytochemically and pharmacologically, which may prove this plant as cheaper and accessible source of valuable drugs.

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Full Length Research Paper

Comparison of hydroxyethyl starch and succinylated gelatin in cadaver renal graft function in kidney-transplant recipients

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Hydroxyethyl starch solution (HES) is increasingly used during kidney transplantation for compensation of hypovolemia. However, the advantages of HES remain unclear. This clinical trial compared the effect of administering 6% HES 130/0.4 and 4% succinylated gelatin (SG) for volume replacement on the rate of delayed graft function (DGF), the occurrence of sensitive markers of renal impairment, and peritransplantation indexes of hemodynamic stability. After exclusion, 71 patients aged 23 to 64 years who underwent cadaver kidney transplantation were enrolled. Of these, 36 received 6% HES 130/0.4 and 35 were given 4% SG for volume replacement. The rate of DGF; the levels of beta 2-microglobulin (β_2 -MG), alpha 1-microglobulin (α_1 -MG), blood urea nitrogen (BUN), and serum creatinine (sCr); and the peritransplantation urine volume were determined and used as primary investigative parameters. The HES group showed a significant postoperative decrease in serum β_2 -MG, BUN, and sCr. Postoperative urine levels of β_2 -MG and α_1 -MG also decreased significantly in patients who received HES ($p < 0.001$) and postoperative urine volume was significantly higher in the HES group as compared to the SG group ($p = 0.0012$). No significant hemodynamic stability differences were seen between patients who received HES or SG colloids; however, 6% hydroxyethyl starch 130/0.4 led to better recovery outcomes in patients who underwent cadaver kidney transplantation.

Key words: Hydroxyethyl starch, succinylated gelatin, volume replacement, renal function, kidney transplantation.

INTRODUCTION

Kidney transplantation has long been the ultimate therapy for end-stage renal disease. However, patients undergoing kidney transplantation can have a wide variety of perioperative complications that are contributing risk factors for transplant failure, including hemodynamic instability, acid-base and electrolyte disturbances, acute renal allograft rejection from impaired renal function, and co-morbid diseases (Stockall et al., 1999). Monitoring of relative or absolute intravascular volume is imperative

during surgery to manage regulation of anesthesia and control blood loss. Pre-operative hemodialysis can affect the recovery of the transplanted kidney. Therefore, maintenance of intravascular volume during kidney transplantation is of crucial importance to ensure optimal graft perfusion and function (Wu et al., 2010).

There is not yet a consensus regarding the optimal plasma substitute for treating hypovolemia in renal transplant patients. Practitioners continue to debate

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whether crystalloids or colloids should be used, and further, if a colloid is used, questions remain regarding which colloid option is most appropriate. When considering volume replacement strategies in renal transplant patients, physicians should balance considerations related to influences on renal function, potential adverse effects, and systemic hemodynamics.

A commonly available colloid option is the poly-dispersed polypeptide produced by degradation of bovine collagen, namely 4% succinylated gelatin (SG; brand name Gelofusine®). The volume-expanding power of SG solution is comparable to that of hydroxyethyl starch, but SG has a mean molecular weight (MW) of less than 30 kDa; SG is considered safe with regards to coagulation and organ function, including kidney function (Van der Linden et al., 2004). SG which has an effective duration of no more than 2 h, is commonly used for volume substitution and has been well-documented as not producing deleterious effects on renal function (Van der Linden et al., 2004; Alavi et al., 2012; Godet et al., 2008).

The colloid hydroxyethyl starch (HES; that is, Voluven®) is a widely used plasma substitute with a longer lasting volume effect than crystalloid solutions (Gallandat et al., 2000). HES is a modified natural polymer of amylopectin with volume expansion properties that is hydrolyzed *in vivo* by serum amylase and then excreted by the kidney. However, because of concerns of negative effects on coagulation and renal function (Cittanova et al., 1996; Davidson, 2006; Schortgen et al., 2001; Peron et al., 2001; De Labarthe et al., 2001; Brunkhorst et al., 2008). HES is contraindicated for hypovolemia correction in kidney transplant patients. Traylor and Pearl (1996) found that different HES molecules have different physicochemical properties depending on their MW, molar substitution degree (mole hydroxyethyl residue per mole glucose subunit), and C2/C6 ratio.

Adverse events have been reported for high-MW, highly substituted HES 200/0.6 (Davidson, 2006; Legendre, 1993; Standl, 1996), whereas lower MW and less substituted version may be safer. Hence, the actual influence of HES solutions on renal function remains a topic of debate (Schortgen et al., 2001; Boldt and Priebe, 2003) and a source of confusion. The rate of metabolism and *in vivo* MW are primarily determined by the molar substitution degree and the C2/C6 ratio (Treib et al., 1999). Greater molar substitution is associated with slower breakdown and elimination of the molecule. The types of HES solutions used in the past have tended to have high levels of total substitution and a high ratio of C2/C6 substitution. There are newer HES products with lower levels of substitution and a lower C2/C6 ratio that can be metabolized more quickly and may therefore have less negative effects on the kidney and coagulation. Notably, the latest generation of HES, HES 130/0.4 (MW, 130 kDa; molar substitution, 0.4; 6% Voluven), which has a C2/C6 ratio greater than eight, can be cleared from

the body 23- to 31-times more quickly than the first-generation HES commonly known as hetastarch (Jungheinrich and Neff, 2005). HES 130/0.4 has the best risk/benefit ratio of all available synthetic colloids (Treib et al., 1999; Dieterich, 2003). Despite widespread use of HES 130/0.4 for patients with renal compromise (Jungheinrich et al., 2002; Davidson, 2008; Boldt, 2009), it has not been studied in kidney transplant recipients. For this reason, we designed a study comparing renal function and hemodynamic stability in renal transplant patients managed with 6% HES 130/0.4 versus 4% SG solution.

This prospective study was aimed at comparing the effects of two fluid regimens administered to kidney transplant recipients on the rate of delayed graft function (DGF), defined by the need for dialysis in the first 7 days after transplantation (Perico et al., 2004). Investigated parameters were the levels of beta 2-microglobulin (β_2 -MG), alpha 1-microglobulin (α_1 -MG), blood urea nitrogen (BUN) and serum creatinine (sCr) peritransplantation, using either 6% HES 130/0.4 or 4% SG. We hypothesized that 6% HES 130/0.4 would yield better hemodynamic stability during kidney transplantation and a better recovery of renal graft function than 4% SG.

METHODOLOGY

Patient demographics

This prospective, randomized, double-blind study was conducted on 76 consecutive patients, aged 23 to 64 years, ASA III or IV, scheduled for cadaver kidney transplantation at a university hospital from May, 2006 through December, 2006. Following approval of the hospital Institutional Review Board, written informed consent was obtained from all patients. We investigated the effects of two fluid regimens, 6% HES 130/0.4 versus 4% SG, on renal graft quality using the following criteria: rate of DGF and renal graft rejection in one year, β_2 -MG level in serum and urine, α_1 -MG level in urine, levels of BUN and sCr in blood, urine volume, and hemodynamic stability. Exclusion criteria were severe cardiovascular disease, liver dysfunction, living donor kidney transplantation, re-transplantation, blood coagulation disorders, preoperative anticoagulants, known HES or gelatin allergy, lack of informed consent, or use of general anesthesia.

The following parameters were recorded for donors: age, gender, weight, and cold and warm ischemia duration. Organs were procured according to our standard clinical practice. University of Wisconsin (UW) solution was used for kidney preservation (Mangus et al., 2006).

Study groups and randomization

Randomization used a computer-generated random number table. Recipients were assigned to volume replacement with either 6% HES 130/0.4 or 4% SG. Group assignments were sealed within opaque envelopes. All colloids were covered in identical opaque bags before infusion. Three anesthesiologists participated in the study. One anesthesiologist, unconnected with the clinical care or data collection, prepared colloids for the two groups according to instructions in the sealed envelopes. A second anesthesiologist monitored and recorded heart rate (HR), arterial blood pressure

(ABP), and central venous pressure (CVP) from the initiation of anesthesia (intrathecal injection) until the end of the surgery and on post-operative days (POD) 1 and 3. This anesthesiologist also controlled colloid infusion. A third anesthesiologist performed all spinal-epidural techniques and collected all blood and urine samples. The patients and the second and third anesthesiologists were blinded to group assignment.

Anesthesia

After patients arrived at the operating room, an 18-gauge IV catheter and 20-gauge arterial cannula were inserted, and a right internal jugular double-lumen central line was placed under local anesthesia to infuse fluid regimens and to monitor CVP. Initial CVP was 5 to 8 mmHg, likely due to dialysis before surgery and relative intravascular hypovolemia secondary to chronic hypertension in these patients. Intraoperative monitoring included electrocardiography, pulse oximetry, ABP and CVP.

All patients received combined spinal-epidural anesthesia performed using a double-space technique in the right lateral decubitus position. Full aseptic precautions were followed. For epidural anesthesia, an 18-gauge Tuohy needle was used to locate the epidural space using loss-of-resistance to air at the T₁₂-L₁ interspace. A 20-gauge epidural catheter was advanced through the needle in a cephalad direction for 3 to 4 cm into the epidural space. The catheter was gently aspirated and checked for the presence of blood or cerebrospinal fluid. A test dose of 3 ml of 2% lidocaine was given through the epidural catheter, followed by a loading dose of 6 ml of 0.75% ropivacaine after 1.5 h. For spinal anesthesia, after location of the epidural space with an 18-gauge Tuohy needle at the L₃-L₄ interspace, dural puncture was performed with a 25-gauge Whitacre needle. Free flow of cerebrospinal fluid was verified and a spinal injection of 10 to 12.5 mg hyperbaric bupivacaine (5 mg/ml) was administered. After removing the spinal needle, the patient was immediately turned to the supine position on the operating table, in a slight head-up tilt position. Testing of pinprick analgesia and motor block was performed at 1, 3, 5, 10 and 15 min; all patients developed a good sensory and motor block. Surgery commenced when the sensory block reached the T₆ level.

Intraoperative patient management

After intrathecal injection, hypotension (a decrease in the mean arterial pressure >30% below baseline) was treated with ephedrine of 6 to 10 mg. During the period from reperfusion until the end of surgery, the patient's systolic blood pressure (SBP) was strictly controlled in the range of 145 to 180 mmHg regardless of the baseline SBP, which was considered beneficial for perfusion of the new kidney. If hypotension, defined as SBP <145 mmHg, occurred after reperfusion, fluid loading was initiated, with 6 to 10 mg ephedrine administered to increase SBP after fluid loading if necessary. If SBP could not be maintained within the target range after up to three administrations of an ephedrine bolus, dopamine infusion at 3 to 10 µg/kg/min was given after communication with the surgeon. Atropine of 0.3 to 0.5 mg was administered for bradycardia, defined as heart rate <50 bpm.

Colloid solutions (6% HES 130/0.4 or 4% SG) were administered at the beginning of surgery to maintain CVP at 10 to 15 mmHg during the procedure. Ringer's lactate (RL) was administered in both groups throughout the operation to compensate for fluid loss from sweating, urine, and physiological demands. RL was also used as a solvent for drugs (e.g. antibiotics). During surgery, RL was administered routinely at 300 ml/h in both groups. Temperature was kept constant at 36 to 37°C.

Each recipient was given methylprednisolone 500 mg at the beginning of surgery, furosamide 100 mg and 20% mannitol 250 ml

during vascular anastomosis, and dexamethasone 50 mg before clamp release. The postoperative immunosuppressive protocol was methylprednisolone 500 mg intravenously on POD 1 and 2, mycophenolate mofetil orally at 1500 mg twice per day from POD 1, prednisone orally at 120 mg per day from POD 3 followed by a decrease of 20 mg each day to 20 mg per day for maintenance, and cyclosporin A orally at 6 to 7 mg/kg/day from POD 3, adjusting dosage according to blood concentration.

At the end of surgery, study fluid was discontinued and all patients received an infusion of cycling crystalloid (a mixture of normal saline, glucose, calcium gluconate and potassium chloride prepared by the urinary surgery department) at 500 ml/h around according to urine volume. Hourly urine output was replaced with cycling crystalloid at 1 ml for each ml of urine.

Outcome measurements

Venous blood samples were analyzed for β₂-MG level before anesthesia (T₀), 5 min after clamp release (T₁), at the end of surgery (T₂), and at POD 1 (T₃), 3 (T₄), 7 (T₅), and 14 (T₆). Levels of sCR and BUN before anesthesia and at POD 1, 3, 7, and 14 were also measured. For urine specimens, volume and β₂-MG and α₁-MG were measured before anesthesia and at POD 1, 3, 7, and 14. As markers of renal impairment, β₂-MG, α₁-MG, sCR, and BUN were recorded until postoperative day 14, because the main effects of colloid administration were assumed to occur within 14 days after renal transplantation (Demian et al., 1999). We also investigated the rate of DGF and renal graft rejection within 1 year for both groups. Hemodynamic performances including SBP, DBP, mean arterial pressure (MAP), HR, and CVP were recorded intraoperatively as well as on POD 1 and 3.

Statistical analysis

Quantitative data were presented as mean ± standard deviation (SD) and analyzed using Student's *t*-test. Qualitative data were expressed as percentages and differences were analyzed with χ² test or Fisher's exact test. Data not normally distributed were tested by Kolmogorov-Smirnov test and presented as medians (interquartile range), and analyzed with Wilcoxon U test as appropriate. The software SPSS v15.0 for Windows (SPSS, Chicago, IL) was used for statistical analysis. A (two-sided) *p*-value less than 0.05 was considered statistically significant.

RESULTS

Patient demographics

Of the 76 consecutive renal transplant patients initially identified, required records were missing for one patient, information about colloid use was incomplete for two patients, one patient died of acute myocardial infarction during surgery, and one patient died of ventricular fibrillation at POD 3, leaving 71 patients for analysis. Characteristics of kidney donors and transplant recipients are in Tables 1 and 2. No significant differences were observed in donor groups for age, gender, weight and cold and warm ischemia duration (Table 2). Demographic characteristics of recipients were similar in the two groups. The HES group received 818 ± 177 ml colloids and the gelatin group received 850 ± 196 ml colloids

Table 1. Recipient characteristics for each colloid group.

Parameter	Group HES (n = 36)	Group gelatin (n = 35)
Age (y)	47 ± 13	45 ± 12
Gender ratio (male/female)	20/16	18/17
Weight (kg)	59 ± 10	62 ± 11
ASA physical status [n (%)]		
ASA III	28 (77.8)	29 (82.9)
ASA IV	8 (22.2)	6 (17.1)
Operation time (min)	185 ± 21	176 ± 19
Volume replacement during surgery (ml)		
Colloids	818 ± 177	850 ± 196
Crystalloids	1070 ± 265	932 ± 236
No. (%) patients given ephedrine bolus after reperfusion	26 (72.2)	24 (68.6)
Total ephedrine dose (mg)	26 (13.5, 26)	26 (8.5, 26)
No. (%) patients given dopamine infusion after reperfusion	10 (27.8)	9 (25.7)
Total dopamine dose (mg)	21 ± 8	20 ± 7

Data are mean ± SD, number (%), or median (interquartile range). HES, hydroxyethyl starch; ASA, American Society of Anesthesiologists; Characteristics of the recipients undergoing kidney transplantation, time of operation and the dosage of colloids therapy as well as administration of vasoactive agents during the operation. No significant differences between two groups.

Table 2. Donor characteristics for each colloid group.

Parameter	Group HES (n = 28)	Group gelatin (n = 27)
Age (y)	39 ± 15	37 ± 13
Gender ratio (male/female)	27/1	27/0
Weight (kg)	67 ± 14	71 ± 15
Cold ischemia duration (min)	378 ± 122	367 ± 114
Warm ischemia duration (min)	7 ± 3	7 ± 2

Data are mean ± SD, or number. HES, hydroxyethyl starch. Characteristics of the donors of cardiac death (DCD) and the mean duration of cold ischemia and warm ischemia. No significant difference between the two groups.

during surgery; these differences were not significant (Table 1).

Hemodynamic stability and DGF rate

Both HES and SG groups were hemodynamically stable during surgery (Table 3). No differences were observed between groups in the dose or number of patients requiring ephedrine bolus and dopamine infusion to maintain SBP within the target range after reperfusion (Table 1).

Blood products were transfused when hemoglobin levels were lower than 80 g/L; this parameter was not different between the groups.

The rate of DGF was 5.56% in recipients receiving 6% HES 130/0.4 compared with 8.57% in those receiving 4% SG ($p = 0.6737$). The rate of renal graft rejection in one year was 5.56% in HES group versus 11.43% in SG

group ($p = 0.4290$).

Outcome measurements in serum

After the surgery, all sensitive markers of renal impairment decreased significantly. The levels of serum β_2 -MG at POD 3, 7 and 14 were significantly lower ($p = 0.0276$, $p < 0.0001$, and $p < 0.0001$, respectively) in the HES group as compared to the SG group (Table 4). Significant decreases were found in the BUN levels at POD 1, 3 and 7 in the HES group when compared with the SG group ($p = 0.0201$, $p < 0.0001$, and $p = 0.0134$, respectively) but no differences were observed at POD 14 (Table 5).

Levels of sCR were significantly decreased in the HES group from POD 1, 7, and 14 compared to the SG group ($p = 0.0087$, $p = 0.0069$, and $p < 0.0001$) (Table 6).

Table 3. Hemodynamics in group HES and group gelatin at five time points (T0–T4).

Hemodynamic parameter	T0	T1	T2	T3	T4
SBP (mmHg)					
Gelatin	187 ± 24	163 ± 16	159 ± 14	155 ± 14	151 ± 13
HES	184 ± 26	165 ± 14	161 ± 13	151 ± 12	155 ± 12
DBP (mmHg)					
Gelatin	102 ± 15	88 ± 14	86 ± 12	87 ± 10	84 ± 9
HES	100 ± 16	84 ± 12	85 ± 10	82 ± 11	83 ± 11
MAP (mmHg)					
Gelatin	130 ± 17	113 ± 14	110 ± 12	109 ± 13	106 ± 10
HES	128 ± 15	110 ± 13	111 ± 14	105 ± 11	107 ± 12
HR (bpm)					
Gelatin	92 ± 11	88 ± 6	90 ± 7	90 ± 6	85 ± 5
HES	98 ± 12	84 ± 5	80 ± 7	83 ± 6	84 ± 8
CVP (mmHg)					
Gelatin	6 ± 2	12 ± 3	13 ± 4	11 ± 3	12 ± 3
HES	7 ± 3	13 ± 3	12 ± 3	12 ± 4	11 ± 2

T₀, Before anesthesia; T₁, 5 min after clamp release; T₂, end of surgery; T₃, first post operative day (POD); T₄, third POD; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HES, hydroxyethyl starch; HR, heart rate; CVP, central venous pressure. Data are mean ± SD. Hemodynamic stability was monitored with BP, HR, and CVP during the operation until the third POD and no significant differences between two groups were observed.

Urine outcome measurements

Levels of β_2 -MG and α_1 -MG before anesthesia in urine could not be precisely determined because they were much higher than the normal range, because of renal failure; however, levels decreased over time after kidney transplantation. Urine β_2 -MG in the HES group as compared to the SG group decreased significantly at POD 3, 7, and 14 ($p = 0.0003$, $p = 0.0002$, and $p < 0.0001$) (Table 7). Urine α_1 -MG in the HES group when compared with the SG group was significantly lower at POD 1, 3 and 14 ($p = 0.0004$, $p = 0.0018$, and 0.0002) (Table 8). The average urine output was abnormal at baseline in both groups and increased in both groups at POD 1, 3, and 7, with no differences between the groups. However, urine output in the HES group was significantly higher than the SG group at POD 14 ($p = 0.0012$); although both groups had values in the normal range (Table 9).

DISCUSSION

This clinical study was designed as a prospective, randomized, double-blinded trial in renal transplantation surgery to assess efficacy of 6% HES 130/0.4 and 4% SG for perioperative hemodynamic stability and renal graft function improvement. Our study demonstrated that

the administration of a colloid solution containing 6% HES 130/0.4 was associated with lower levels of serum β_2 -MG, BUN and creatinine, and lower levels of urine β_2 -MG and α_1 -MG than administration of 4% gelatin. Optimization of intravascular volume during kidney transplantation is known to be crucial for hemodynamic stability and renal graft function (Wu et al., 2010). Since 4% SG is the standard for volume substitution, with a well-documented lack of deleterious effects on renal function (Van der Linden et al., 2004; Alavi et al., 2012; Godet et al., 2008). it is clearly justified as the reference treatment.

Concerns about the adverse effects of HES on renal function continue. Available data largely justified this concern and systematic studies have addressed this question (Schortgen et al., 2001; Boldt et al., 1993; Kumle et al., 1999; Hüter et al., 2009). Cittanova et al. (1996) investigated prospectively whether hydroxyethyl starch use in brain-dead kidney transplant donors was a determinant of renal function in the organ recipient after transplantation. When 27 kidney donors were randomized to either a hydroxyethyl starch-gelatin regimen for fluid management, or to a gelatin-only regimen, a significantly higher proportion in the hydroxyethyl starch group required hemodialysis or hemodiafiltration in the first 8 days after transplantation compared to the gelatin group (33% versus 5%; $p = 0.029$). In addition, at 10 days after transplantation, mean (SD) sCR concentrations were

Table 4. Comparison of serum β_2 -MG between group HES and group gelatin at seven time points (T0–T6).

Group	Serum β_2 -MG ($\mu\text{g/ml}$), median (interquartile range)						
	T0	T1	T2	T3	T4	T5	T6
Gelatin (n = 35)	6.68 (4.82, 8.46)	5.67 (4.40, 7.28)	5.65 (3.22, 7.52)	3.78 (2.02, 5.50)	3.23 (1.84, 5.45)	2.18 (1.46, 4.97)	3.36 (2.08, 6.24)
HES (n = 36)	5.58 (2.88, 9.41)	5.50 (4.42, 6.46)	5.40 (4.49, 6.37)	3.66 (2.87, 5.86)	2.23 (1.79, 2.95)	1.27 (1.01, 1.77)	1.65 (1.15, 2.11)
Z	0.5638	0.2531	0.0633	-0.6442	2.2027	3.9627	4.6758
P	0.5729	0.8002	0.9495	0.5194	0.0276*	<0.0001*	<0.0001*

T₀, Before anesthesia; T₁, 5 min after clamp release; T₂, end of surgery; T₃, first postoperative day (POD); T₄, third POD; T₅, seventh POD; T₆, fourteenth POD; HES, hydroxyethyl starch. Serum β_2 -MG levels decreased in both groups postoperatively and they were significantly lower in the HES group at POD 3, 7 and 14 vs. SG group (* $p < 0.05$).

Table 5. Comparison of serum BUN between group HES and group gelatin at five time points (T0, T3–T6).

Group	Serum BUN (mmol/L), median (interquartile range)				
	T0	T3	T4	T5	T6
Gelatin (n = 35)	18.7 (15.2, 23.9)	10.0 (7.6, 15.3)	5.7 (4.1, 7.3)	7.2 (4.5, 9.5)	5.4 (3.6, 8.2)
HES (n = 36)	19.2 (13.40, 24.05)	7.40 (6.35, 12.15)	3.35 (2.60, 4.45)	4.70 (3.80, 6.65)	5.5 (4.6, 6.4)
Z	0.3106	2.3240	3.9522	2.4736	0.0460
P	0.7561	0.0201*	<0.0001*	0.0134*	0.9633

T₀, Before anesthesia; T₃, first postoperative day (POD); T₄, third POD; T₅, seventh POD; T₆, fourteenth POD; HES, hydroxyethyl starch. BUN levels decreased postoperatively in both groups and dropped significantly at POD 1, 3 and 7 in the HES group vs. the SG group (* $p < 0.05$), but there was no difference at POD 14.

significantly higher in recipients of kidneys of donors randomized to the hydroxyethyl starch group as compared to the gelatin group (312 [259] $\mu\text{mol/L}$ versus 145 [70] $\mu\text{mol/L}$; $p = 0.009$). However, Deman et al. (1999) failed to confirm these findings in a retrospective, multicenter analysis of kidney transplant recipients that used DGF as the main outcome measure. They found that 3 of 20 recipients (15%) in the HS-group had DGF that required treatment with dialysis when compared with 14 of 73 (19.2%) in the gelatin-albumin group ($p = 0.450$). This was in accordance with our findings (5.56% in HES group versus 8.57% in gelatin group). Interestingly, our study found that the rate of renal graft rejection at

one year remained lower in the HES group than the SG group (5.56% versus 11.43%). However, this remains to be verified.

In kidney transplant patients, osmotic nephrosis was found after receiving HES that is less easily metabolized (HES 200/0.62) (Cittanova et al., 1996; Pillebout et al., 2005). However, the lesions had no significant effect on the occurrence of DGF and serum creatinine at three and six months post transplantation. In addition, osmotic-like lesions of the tubules were found in the patients who received RL (Hüter et al., 2009). Thus, whether the tubular epithelial vacuoles represent renal injury or are an epiphenomenon without clinical importance remains to be determined. Adverse

effects of HES are considered to occur only after higher doses and prolonged usage (Treib et al., 1999). Our findings corroborate prior studies showing that 6% HES 130/0.4-based volume therapy is superior to gelatin administration for maintaining kidney function. In patients undergoing abdominal aortic aneurysm surgery, 6% HES 130/0.4 maintained glomerular and tubular function better than 4% SG over the subsequent five PODs (Mahmood et al., 2007). Hüter et al. (2009) concluded that 10% HES 200/0.5 has a greater pro-inflammatory effect than 6% HES 130/0.42 and causes more pronounced tubular damage than 6% HES 130/0.42 and RL. This result indicated that 6% HES 130/0.42 has a

Table 6. Comparison of serum creatinine between group HES and group gelatin at five time points (T0, T3–T6).

Group	Serum creatinine (mmol/L), median (interquartile range)				
	T0	T3	T4	T5	T6
Gelatin (n = 35)	825.6 (729.3, 1084.9)	468.7 (342.5, 576.6)	223.7 (145.2, 576.6)	161.2 (137.0, 246.6)	133.7 (120.7, 219.8)
HES (n = 36)	772.75 (684.7, 1086.85)	346.45 (247.05, 496.55)	186.00 (151.55, 242.85)	143.85 (117.00, 155.55)	100.00 (76.05, 119.95)
Z	0.5003	2.6224	0.7821	2.7029	4.9514
P	0.6169	0.0087*	0.4342	0.0069*	<0.0001*

T₀, Before anesthesia; T₃, first postoperative day (POD); T₄, third POD; T₅, seventh POD; T₆, fourteenth POD; HES, hydroxyethyl starch. Serum creatinine levels dropped postoperatively in both groups and were significantly decreased in the HES group vs. the SG group at POD 1, 7, and 14 (**p* < 0.05) but no difference was observed at POD 3.

Table 7. Comparison of urine β_2 -MG between group HES and group gelatin at five time points (T0, T3–T6).

Group	Urine β_2 -MG ($\mu\text{g/ml}$), median (interquartile range)				
	T0	T3	T4	T5	T6
Gelatin (n = 35)	>10 (>10, >10)	8.98 (7.38, >10)	8.36 (7.21, 10.1)	7.05 (5.65, 9.87)	4.72 (3.37, 8.25)
HES (n = 36)	>10 (>10, >10)	8.98 (6.73, 9.86)	6.44 (4.57, 7.45)	5.40 (3.79, 6.35)	2.4 (1.95, 3.71)
Z	0.0000	3.6025	3.6025	3.6637	4.0257
P	>0.9999	0.5345	0.0003*	0.0002*	<0.0001*

T₀, Before anesthesia; T₃, first postoperative day (POD); T₄, third POD; T₅, seventh POD; T₆, fourteenth POD; HES, hydroxyethyl starch. Urine β_2 -MG levels could not be precisely determined before anesthesia since they were much higher than the normal range because of renal failure. However, urine β_2 -MG levels in both groups decreased over time after kidney transplantation. Urine β_2 -MG levels were decreased significantly in the HES group vs. the SG group at POD 3, 7, and 14 (**p* < 0.05).

Table 8. Comparison of urine α_1 -MG between group HES and group gelatin at five time points (T0, T3–T6).

Group	Urine α_1 -MG in $\mu\text{g/ml}$, median (interquartile range)				
	T0	T3	T4	T5	T6
Gelatin (n = 35)	>800 (>800, >800)	>800 (765.54, >800)	>800 (>800, >800)	>800 (749.56, >800)	>800 (723.44, >800)
HES (n = 36)	>800 (>800, >800)	704.85 (655.55, >800)	>800 (631.01, >800)	>800 (618.30, >800)	617.31 (472.06, >800)
Z	0.0000	3.5746	3.1226	0.8126	3.7207
P	>.9999	.0004*	.0018*	.4164	.0002*

T₀, before anesthesia; T₃, first postoperative day (POD); T₄, third POD; T₅, seventh POD; T₆, fourteenth POD; HES, hydroxyethyl starch. Urine α_1 -MG levels could not be precisely determined before anesthesia since they were much higher than the normal range because of renal failure. However, urine α_1 -MG levels decreased over time in both groups after kidney transplantation. Urine α_1 -MG was significantly lower in the HES group vs. the SG group at POD 1, 3, and 14 (**p* < 0.05).

Table 9. Comparison of urine volume between group HES and group gelatin at five time points (T0, T3–T6).

Group	Urine volume in ml, median (interquartile range)				
	T0	T3	T4	T5	T6
Gelatin (n = 35)	500 (300, 700)	16200 (13900, 18500)	13000 (10700, 14100)	6300 (3400, 7200)	2700 (2500, 3300)
HES (n = 36)	500 (350, 675)	16600 (15700, 17850)	12450 (9750, 14100)	6600 (5250, 7350)	3450 (2950, 3750)
Z	0.3638	-0.3854	.2437	-0.9264	-3.2283
P	.7160	.7000	.8047	.3542	.0012*

T₀, Before anesthesia; T₃, first postoperative day (POD); T₄, third POD; T₅, seventh POD; T₆, fourteenth POD; HES, hydroxyethyl starch. The average urine output was abnormally low at baseline in both groups and increased in both groups at POD 1, 3, and 7, with no difference between the groups. Urine output was significantly higher in the HES group vs. the SG group at POD 14 (* $p < 0.05$), although both groups had values in the normal range.

less detrimental effect on renal function than 10% HES 200/0.5. We found that 6% HES 130/0.4 was beneficial for renal graft function when compared with 4% SG. Sensitive markers of renal impairment decreased significantly in both groups and gradually lowered to normal levels over time in the HES group, but remained higher in the SG group. This is the first report to describe the effect of renal function of 6% HES 130/0.4 on kidney transplant recipient and is also the first time to demonstrate its benign outcome on renal graft function.

UW solution is the gold standard preservation solution for cadaveric liver, kidney, pancreas, and small bowel transplantation (Mangus et al., 2006). UW solution is designed to reduce the physiological and biochemical effects of ischemia from cold storage. The main purpose of UW is to prevent cell swelling and interstitial edema formation by including substances that are osmotically active and impermeable to cells (Morariu et al., 2003).

A major component of UW is HES (via starch), a colloid used to prevent hypothermic-induced cell damage during major surgery. HES decreases transvascular fluid flux and edema formation, maintains colloid osmotic pressure, and preserves the microvascular barrier (Adam et al., 1990).

These findings support our hypothesis that HES has a positive effect on renal graft function.

Our study has limitations. The low rate of DGF demonstrated an extremely short warm ischemia time, which is important for renal graft function. Also, our small sample size has limited power to detect meaningful differences in outcomes. Additional study is needed in large-scale, multi-center trials. Also needed are comparisons of recipient renal functions from a single cadaver kidney, as in Blasco et al. (2008). In conclusion, the influence of different intravascular volume regimens on renal function still remains controversial. When weighing the possible deleterious influence of HES on kidney grafts, the type of HES, duration time, and total volume must be considered. In our prospective study, a modern, third-generation, rapidly degradable HES preparation with a low degree of substitution (6% HES 130/0.4) was associated with better effects on the renal function of the kidney transplant recipients than SG.

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Full Length Research Paper

Evaluation of the phenolic and flavonoid contents and radical scavenging activity of three southern African medicinal plants

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Warburgia salutaris (Bertol. F.) Chiov's, *Rhoicissus tridentata* (L.f.) Wild & Drum and *Terminalia sericea* (Burch. ex DC.), are widely used medicinal plants in southern Africa. The aim of the study was to determine the phenolic and flavonoid content and evaluate the antioxidant activity of the three medicinal plants. Total phenolic and flavonoid contents were determined spectrophotometrically as gallic acid and rutin equivalents, respectively. Individual phenolic acids were identified by means of gas chromatography-mass spectrometry. Antioxidant activities of the crude extracts were assessed using the TEAC assay. The highest phenolic content was detected in the crude methanol extract of the bark of *W. salutaris* and the highest flavonoid content was found in the crude methanol extract of the leaves of this plant. In all the studied plants the alkaline hydrolysable fraction yielded a greater variety of phenolic acids compared to the soluble/free phenolic acid fractions. The three medicinal plants investigated were found to be strong radical scavengers supporting the traditional use of these medicinal plants.

Key words: Antioxidant activity, flavonoids, medicinal plants, phenolic acids, phenolic content, *Rhoicissus tridentata*, *Warburgia salutaris*, *Terminalia sericea*.

INTRODUCTION

The use of medicinal plants forms the basis of the traditional healing system in many developing countries. The plants investigated in this study, *Warburgia salutaris* (Bertol. F.) Chiov's (Canellaceae), *Rhoicissus tridentata* (L.f.) Wild & Drum (Vitaceae) and *Terminalia sericea* (Burch. ex DC.) (Combretaceae), are widely used medicinally. *W. salutaris* is commonly employed in the treatment of inflammatory diseases as well as coughs, colds and chest complaints (Watt et al., 1962; Hutchings, 1996). *R. tridentata* tubers are used for gynaecological purposes,

stomach ailments, as well as kidney and bladder complaints (Watt et al., 1962; Hutchings, 1996; Brookes et al., 2006). *T. sericea* root decoctions are used to treat pneumonia, stomach disorders and diarrhoea (Watt et al., 1962; Hutchings, 1996).

Medicinal plants contain many compounds, amongst which are polyphenols that possess antioxidant properties against oxidative stress, a major cause and aggravating factor in a number of chronic diseases (Ray et al., 2000). Flavonoids have been reported as being able to

scavenge basically all known reactive oxygen species (ROS) (de Beer et al., 2002). These compounds show antioxidative properties by a number of mechanisms, including scavenging of free radicals, chelation of metal ions such as iron and copper which are of major importance in the initiation of radical reactions, and inhibition of enzymes responsible for free radical generation (Ebenharder et al., 2003).

Phenolic acids are a subgroup of secondary metabolites that are commonly found in plants and are generally divided into two groups. Depending on the core structure they could be classified as either hydroxybenzoic acid derivatives (gallic, ellagic, vanillic and syringic acids) or hydroxycinnamic acid derivatives (*p*-coumaric, ferulic, caffeic, and synaptic acids). The hydroxycinnamic acid derivatives are more potent antioxidants than their hydroxybenzoic acid counterparts. This is due to an increase in the possibilities for delocalisation of the phenoxyl radical by hydroxycinnamic acid derivatives (Chen et al., 1997). Not only do phenolic acids possess antioxidant activity because of their stable radical intermediates, they are also able to donate hydrogen and electrons to stabilise other free radicals. This is evident from the fact that an increased number of hydroxylated positions on the core structure usually corresponds with more potent antioxidant activities (Rice-Evans et al., 1996; de Beer et al., 2002).

Although *W. salutaris* (Bertol. F.) Chiov., *R. tridentata* (L.f.) Wild & Drum and *T. sericea* (Burch. ex DC.) have been reported to be biologically active, the compounds responsible for the activity have not been well-researched. The aim of this study was to determine the total phenolic and flavonoid contents, identify the individual phenolic acids and assess the antioxidant activities of the three above-mentioned medicinal plants.

MATERIALS AND METHODS

Chemicals, solvents and reagents

All solvents and reagents from various suppliers were of the highest purity. Folin-Ciocalteu phenol reagent, gallic acid, rutin, methanol (99.9%, HPLC grade), ethyl acetate, diethyl ether and all phenolic acid standards; vanillic, ferulic, caffeic, syringic, *p*-coumaric, sinapic, protocatechuic, 3-hydroxybenzoic, 4-hydroxybenzoic, 2-hydroxycinnamic, 3-hydroxycinnamic, gallic acids; bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), 6-hydroxy-2,5-7-8-tetramethylchroman-2-carboxylic acid (Trolox), ethanol, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and potassium persulfate were purchased from Sigma-Aldrich. Sodium carbonate (Na₂CO₃) and aluminum chloride (AlCl₃) were purchased from Merck. In all experiments, purified deionised water used was obtained from a MilliQ water purification system (Millipore).

Plant material

Bark of the *W. salutaris*, was purchased from the Mai Mai market, Johannesburg, South Africa, tubers of *R. tridentata* were a gift from

Dr. Lynn Katsoulis (Department of Pharmacology, University of the Witwatersrand, South Africa) and roots of *T. sericea* were collected in Makhado, Limpopo Province, South Africa. The identities of the specimens were confirmed by a botanist and voucher specimens of the plants are deposited in the Soutpansbergensis herbarium in Makhado. Plant material was dried and ground to a fine powder using an Ika Analytical Mill (Staufen, Germany).

The ground material of the different parts of these plants were used to prepare crude methanol and water extracts and used for the determination of their total flavonoid and phenolic acid contents as well as for the assessment of their ability to scavenge the ABTS^{•+}, the pre-formed radical monocation of ABTS. The ground material of these plants was also used to prepare free and bound phenolic acid extracts followed by their identification with gas chromatography-mass spectrometry (GC-MS).

Crude extracts

The extraction procedure for total phenolic compounds in methanol extracts was according to Velioglu et al. (1998) with minor modifications. One gram of the ground plant material was shaken for 4 h at 25°C with 99.9% methanol (10 ml). The mixture was centrifuged (1 000 g; 15 min) and the supernatant filtered through 0.45 µm membrane filters (Millipore). Purified deionised water (15 ml) was added to the supernatant and the methanol evaporated at 30°C. Ethyl acetate (5 ml) was added to perform two liquid-liquid extractions of the aqueous phase. Ethyl acetate layers were combined and anhydrous sodium sulphate added to suppress water. The ethyl acetate layers were filtered, concentrated to dryness using a rotary evaporator and the residue re-dissolved in 5 ml ethanol. Yields were determined gravimetrically.

The method of Yu et al. (2001) was followed in order to extract total phenolic compounds from aqueous extracts. One gram of ground plant material was suspended in 10 ml of boiling purified deionised water, heated for 30 min, after which the mixture was allowed to cool to room temperature. The latter was centrifuged (10 000 g; 15 min) and filtered (0.45 µm). The filtrate was extracted three times with 5 ml ethyl acetate. All the ethyl acetate fractions were combined and anhydrous sodium sulphate added to suppress water. The ethyl acetate fraction was filtered, dried and the residue re-dissolved in 5 ml purified deionised water.

Quantitation of total flavonoid and phenolic compounds in methanol and water crude extracts

Total flavonoid content: Flavonoid content was determined using the method of Quettier-Deleu et al. (2000). A 1 ml of the undiluted extract was added to 1 ml of a 2% aluminium chloride reagent and incubated in the dark for 10 min. The absorbance was read at 430 nm using a Spectronic Genesys 5 spectrophotometer. Flavonoid content expressed in mg/g as rutin equivalent (RE) was calculated using the equation:

$$X = \frac{[EX \text{ Abs} \times (\text{mg rutin in 1 ml ethanol}) \times \text{DiF.} \times \text{FW/DW}]}{[\text{ST Abs} \times \text{weight of plant extract}]}$$

Where: X = Flavonoid content, mg/g plant extract in rutin equivalent; DiF. = Dilution factor of the extract; FW/DW = the ratio between the fresh and dry plant weight (water percentage); EX Abs = Absorbance of the plant extract; St Abs = Absorbance of the rutin standard.

Total phenolic content: Total phenolic content in methanol and water extracts was determined using the Folin-Ciocalteu method of Djeridane et al. (2006). A gallic acid standard curve was established

(0.1 to 0.5 mg/ml). One hundred microlitres of the sample was dissolved in 500 μ l of diluted (1/10) Folin-Ciocalteu phenol reagent and 1000 μ l of purified deionised water. The solution was mixed and allowed to stand at room temperature for 3 min. Thereafter, a 1.5 ml of a 20 % Na_2CO_3 solution was added and the mixture was shaken and incubated for 2 h in the dark. Absorbance was read at 765 nm using a Spectronic Genesys 5 spectrophotometer. Total phenolic content of methanol and boiling water extracts, expressed as gallic acid equivalence (GAE) or milligram of gallic acid per gram dry weight of plant material, were then calculated using the following formula:

$$C = (c \times V) / m$$

where: *C* is the total phenolic content in mg/g plant extract, in GAE; *c* is the concentration of Gallic acid established from the calibration curve in mg/ml; *V* is the volume of extract in ml; *m* is the weight of plant methanolic or boiling water extract in g.

Antioxidant activity

Antioxidant activity was determined according to the method of Re et al. (1999). This method involves the production of the ABTS^{•+} radical through the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate. ABTS^{•+} was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm. ABTS^{•+} (2 ml) was added to 20 μ l of different concentrations of plant extract (1 to 4 mg/ml), and incubated for 3 min, after which the absorbance was determined at 734 nm (PerkinElmer Lambda 25). Trolox was used as positive control. TEAC values were calculated according to Obón et al. (2005) where the ratio of the slope of the corresponding sample versus the calculated slope for Trolox was determined.

Phenolic acid extraction and identification

Extraction and hydrolysis was performed according to the method of Kim et al. (2006). Plant material was defatted twice with *n*-hexane at 4:1 ratio (v/w) to plant material, shaking for 1 h at 25°C and filtering through a Whatman No. 1 filter paper. The defatted fraction was extracted twice with methanol for 5 h while shaking at 25°C, filtered through Whatman No. 1 filter paper and the supernatants dried using a rotary evaporator. Soluble/free phenolic acids were extracted from the residue whereas the plant residue remaining on the filter paper was used to extract bound phenolic acids.

Free phenolic acids

The dried supernatant residue from the methanol extract was re-dissolved in acidified water (pH 2, using HCl) and partitioned twice for 1 h each with diethyl ether-ethyl acetate (1:1). The collected diethyl ether-ethyl acetate extracts were dehydrated with anhydrous sodium sulphate and filtered (0.45 μ m). Sample solvent was evaporated under nitrogen stream and then derivatized by the silylation of the hydroxyl groups in plant phenolic acids using 250 μ l BSTFA containing 1% TMCS at 80°C for 30 min.

Bound phenolic acids

The residue remaining on the filter paper was hydrolyzed with 4 M NaOH for 4 h at 25°C. The supernatants were collected and acidified (pH 2) using 6 M HCl and the solution extracted using diethyl ether-ethyl acetate (1:1). Sample solvents were dried and derivatized as described earlier.

Phenolic acid identification

A Trace GC 2000 was coupled to a Trace MS in the Electron Impact (EI) mode with the electron energy set at 70 eV and the mass range at *m/z* 25-700. The column used was a Zebron capillary column ZB-5MS (30 m \times 0.25 mm, i.d.) with 0.25 μ m film thickness (Phenomenex, USA). The injector temperature was set at 280°C and the detector at 290°C. Analysis was performed in the split-less mode with 1 min split-less-time. The gradient temperature program was set as follows: 100°C held for 5 min, increasing 5°C/min to a final temperature of 280°C held for 5 min. The post run was 10 min at 100°C. The flow rate of carrier gas (helium) was maintained at 1.2 ml/min. X-calibur software was used for data acquisition. Identification of individual phenolic acids in each plant extract was established by comparing their gas chromatographic retention times and silylated derivative mass spectra to those of purified standards.

Statistical analysis

Results are expressed as mean \pm the 95% confidence interval as obtained from triplicates run on three independent analyses. The data was analysed using GraphPad Prism 4.0 and STATA software packages.

RESULTS AND DISCUSSION

Phenolic and flavonoid content of the crude water and methanol extracts of the three plants are provided in Table 1. With regards to the water extracts, roots of *T. sericea* contained the highest flavonoid and phenolic content whereas for methanol extracts, the bark of *W. salutaris* contained the highest total phenolic content. The total phenolic content in all plant extracts was found to be higher in methanol extracts than water extracts. *W. salutaris* has been shown to contain flavonoids and flavonols (Manguero et al., 2003; Frum et al., 2005) and plants from the Vitaceae family are known to contain numerous phenolic compounds (Dictionary of Natural Products, 1996).

Phenolic acids are a subgroup of secondary metabolites that are commonly found in plants, which occur in the free and bound forms. A list of free and bound phenolic acids identified in the three plants is provided in Table 2. It can be seen that in addition to the phenolic acids, organic acids (malic, succinic and fumaric acids) were detected in the free phenolic fraction and malonic and propanoic acids in the hydrolysable fraction of *R. tridentata*. Homovanillyl alcohol, a product of lignin hydrogenation, was also found in the soluble / free phenolic fraction of *R. tridentata*. The presence of both hydroxybenzoates (gallic and vanillic acids) and hydroxyl-cinnamates (ferulic acid) were confirmed in the bound phenolic fraction of all three plants. Only extracts of the roots of *T. sericea* contained hydroxycinnamates in the free phenolic fraction, whereas hydroxybenzoates were found in the free phenolic acid fractions of all extracts. Phenolics belong to a category of natural compounds, which possess antioxidant (Chen et al., 1997; Rice-Evans et al., 1996; Cai et al., 2006) and anti-inflammatory activities

Table 1. Total phenolic (TPC), total flavonoid (TFC) concentrations and antioxidant activities of standard hydroxybenzoic and hydroxycinnamic acids as well as crude methanol and water extracts of the three plants investigated. Slopes indicate those of the relevant percentage inhibition plots with the origin as intercept.

Compound	Slope ($\pm 95\%$ CI)	Antioxidant potency relative to Trolox	TPC (mg/g)	TFC (mg/g)
Hydroxybenzoic acids				
Gallic acid	244.4 \pm 44.4	9.4	-	-
3,4-dihydroxybenzoic acid	191.6 \pm 85.5	7.4	-	-
Syringic acid	83.9 \pm 6.6	3.2	-	-
Hydroxycinnamic acids				
Caffeic acid	105.7 \pm 36.6	4.1	-	-
Sinapic acid	99.1 \pm 11.1	3.8	-	-
<i>p</i> -coumaric acid	96.7 \pm 9.3	3.7	-	-
Ferulic acid	31.7 \pm 7.7	1.2	-	-
2-hydroxycinnamic acid	11.8 \pm 2.2	0.5	-	-
3-hydroxycinnamic acid	0.7 \pm 1.4	0.0	-	-
Vanillic acid	n.d.	n.d.	-	-
Plant extracts				
<i>W. salutaris</i> (leaves) crude MeOH	98.9 \pm 5.8	3.8	12.6 \pm 0.1	3.6 \pm 0.1
<i>W. salutaris</i> (leaves) crude H ₂ O	48.2 \pm 3.7	1.9	6.0 \pm 0.1	1.0 \pm 0.1
<i>W. salutaris</i> (bark) crude MeOH	126.7 \pm 15.1	4.9	13.8 \pm 0.2	0.2 \pm 0.1
<i>W. salutaris</i> (bark) crude H ₂ O	74.2 \pm 3.9	2.9	6.9 \pm 0.1	0.6 \pm 0.1
<i>R. tridentata</i> (tubers) crude MeOH	135.3 \pm 24.8	5.2	11.4 \pm 0.1	0.2 \pm 0.1
<i>R. tridentata</i> (tubers) crude H ₂ O	105.0 \pm 12.4	4.0	6.4 \pm 0.1	0.4 \pm 0.1
<i>T. sericea</i> (roots) crude MeOH	133.9 \pm 27.2	5.2	12.5 \pm 0.1	1.2 \pm 0.1
<i>T. sericea</i> (roots) crude H ₂ O	128.8 \pm 18.6	5.0	9.0 \pm 0.1	6.0 \pm 0.1
Trolox	26.0 \pm 3.2	1	-	-

n.d. = not detected.

(Tunon et al., 2009). The antioxidant activity of crude methanol and water extracts of leaves and barks of *W. salutaris*, of tuber of *R. tridentata*, and roots of *T. sericea* are presented in Table 1. The crude methanol extracts of the roots of *T. sericea* and tubers of *R. tridentata* showed the highest antioxidant activity while the majority of phenolic acid standards had antioxidant activity better than Trolox. Antioxidant activity has previously been reported for the bark of *T. sericea* (Opoku et al., 2002; Steenkamp et al., 2004; Masoko et al., 2005), *W. salutaris* (Leshwedi et al., 2008) and tubers of *R. tridentata* (Naidoo et al., 2006) where the antioxidant activity of the latter plant has in part been ascribed to the compounds: catechin, epicatechin, gallic acid and epigallo-catechin-gallate (Naidoo et al., 2006).

The radical scavenging activity of phenolic acids is related to the number and position of hydroxyl groups and methoxy substituents in the molecules (Cai et al., 2006). For example, gallic acid (3, 4, 5-trihydroxybenzoic acid, with the most hydroxyl groups, had the strongest radical scavenging activity (Table 1). This finding is

supported by Rice-Evans et al. (1996) who reported that gallic acid has increased antioxidant activity relative to Trolox owing to its greater number of hydroxyl groups. Furthermore, gallic acid is a strong chelating agent and forms complexes of high stability with Fe³⁺ ions (Li et al., 2000). This also corresponds with some of the previously reported activities of gallic acid, which include anticancer (Chen et al., 2009) and antibacterial activities (Kang et al., 2008).

Structure-antioxidant activity of the studied phenolic compounds could be observed. For example, the antioxidant response in dihydroxybenzoic acids is reported to be dependent on the relative positions of the hydroxyl groups in the ring. Dihydroxylation in the *ortho* and *meta* or in the *meta* and *para* positions to the carboxylate group enhances antioxidant activity (Rice-Evans et al., 1996). From the results presented in Table 1, it can therefore be seen that 3,4 dihydroxybenzoic acid with hydroxyl groups positioned in the *meta* and *para* positions to the carboxylate group gave high TEAC values. On the other hand, methoxy substitution in

Table 2. Free and bound phenolic acids identified in the roots of *T. sericea*, tubers of *R. tridentata*, bark of *W. salutaris* and leaves of *W. salutaris*. Intensities of fragmentation ions are expressed as percentages of the base peak (In parentheses) [EI, 70 eV].

Herb	Free phenolic acid	Bound phenolic acid	Retention time (min)	Fragmentation ions used to confirm phenolic acids (intensities)	
<i>T. sericea</i> (roots)	Gallic acid		25.56	458.4 (98); 443.0 (51); 281.0 (97)	
	Vanillic acid		21.44	312.0 (61); 297.0 (100); 267.0 (68); 223.1 (54)	
	3,4-dihydroxybenzoic acid		22.63	370.1 (49); 355.1 (28); 281.0 (12); 193.1 (100)	
	p-Coumaric acid		25.09	308.1 (9); 293.1 (17); 219.1 (14)	
	Caffeic acid		28.86	396.1 (81); 381.1 (21); 307.1 (13); 219.1 (99)	
		Gallic acid		25.57	458.5 (96); 443.1 (52); 281.1 (25)
		Vanillic acid		21.43	312.1 (61); 297.2 (100); 267.1 (66); 223.2 (50)
		3,4-dihydroxybenzoic acid		22.62	370.2 (36); 355.2 (20); 193.1 (77)
		p-Coumaric acid		25.08	308.1 (62); 293.1 (81); 219.2 (75)
		Caffeic acid		28.85	396.2 (29); 219.2 (35)
		Ferulic acid		28.01	338.1 (100); 323.1 (62); 293.0 (39); 249.1 (53)
	Syringic acid		24.25	342.1 (53); 327.1 (80); 312.0 (60); 297.0 (52)	
	4-hydroxycinnamic acid		18.35	282.2 (24); 267.1 (100); 223.2 (71); 193.1 (55)	
	Hydrocinnamic acid		21.35	310.2 (26); 192.2 (74); 177.1 (21)	
	Benzoic acid		26.2	428.2 (20); 325.1; (33) 236.2 (44)	
	4-hydrophenylacetic acid		18.59	252.2 (35); 192.2 (10); 179.2 (100); 131.1 (10)	
<i>W. salutaris</i> (bark)	3,4-dihydroxybenzoic acid		22.63	370.1 (56); 355.1 (32); 281.1 (16); 193.1 (100)	
	Vanillic acid		21.44	312.2 (51); 297.1 (92); 267.1 (65); 223.1 (57)	
		Gallic acid	25.47	458.2 (37); 443.2 (14); 281.1 (59)	
	Vanillic acid		21.47	312.1 (59); 297.1 (100); 267.1 (64); 223.1 (53)	
	3,4-dihydroxybenzoic acid		22.67	370.1 (70); 355.1 (42); 281.1 (15); 193.1 (100)	
	Syringic acid		24.27	342.1 (43); 327.1 (66); 312.0 (48); 297.0; (40)	
<i>W. salutaris</i> (leaves)	n.i.	4-hydroxybenzoic acid	18.51	282.3 (10); 267.1 (37); 223.1 (18); 193.0 (17)	
		Syringic acid	24.4	342.2 (27); 327.2 (46); 297.2 (24); 312.2 (31); 253.1 (15)	
		p-Coumaric acid	25.21	308.2 (55); 293.1 (83); 219.1 (71)	
		Ferulic acid	28.15	338.2 (100); 323.2 (63); 308.1 (62); 249.1 (54)	
		Sinapic acid	30.87	368.2 (100); 353.2 (38); 338.2 (95); 279.1 (16)	
<i>R. tridentata</i> (tubers)	Vanillic acid		21.44	312.2 (28); 297.3 (47); 267.2 (39)	
	3,4-dihydroxybenzoic acid		22.63	370.2 (37); 355.2 (21); 281.1 (9); 193.1 (100)	
	Gallic acid		25.5	458.2 (74); 443.2 (47); 281.1 (87)	
	Phthalic acid		19.81	310.3 (4); 295.2 (42); 221.2 (18); 147.1 (100)	

Table 2. Contd.

Vanillic acid	21.44	312.3 (31); 297.3 (58); 267.2 (44)
3,4-dihydroxybenzoic acid	22.64	370.2 (14); 355.2 (8); 281.1 (4); 193.1 (37)
Gallic acid	25.51	458.2 (71); 443.2 (46); 281.1 (79)
Ferulic acid	28.04	338.2 (39) ; 323.2 (25); 308.1 (22); 249.2 (23)
<i>p</i> -coumaric acid	25.09	308.2 (36); 293.1 (52); 219.1 (56)

n.i. = none identified.

hydroxylbenzoates results in a decrease in antioxidant capacity when the 3 and 5 hydroxyl groups in the trihydroxylbenzoic acid (gallic acid) are replaced with methoxy groups in *p*-hydroxydimethoxybenzoic acid (syringic acid). Subsequently, it can be seen from this table that radical scavenging capacity of hydroxybenzoic acids were found to decrease in the following order: gallic acid > 3, 4 dihydroxybenzoic acid > syringic acid. For hydroxycinnamic acids, it was observed that dihydroxyl cinnamates (caffeic acid) were more active in scavenging radicals than monohydroxycinnamates (*p*-coumaric acid; 2-hydroxycinnamic acid). Sinapic acid with methoxy substituents on the 3,5-hydroxyl was also more active than the monohydroxy and the hydroxymethoxy cinnamates (ferulic acid). An increase in antiradical capacity was noted in monohydroxycinnamic acid with the hydroxyl group on the *p*-position (*p*-coumaric acid) compared to the corresponding *m* and *o* positions (2- and 3- hydroxycinnamic acids). Besides the number and position of hydroxyl groups and methoxy substituents in hydroxycinnamates, the acrylic acid side chain (-CH=CH-COOH) in hydroxycinnamates is considered to be key for the significantly greater antioxidant efficiency than the carboxylic acid side chain (-COOH) in the hydroxybenzoates (Rice-Evans et al., 1996). An overall view of the antioxidant activity of phenolic

acids indicates that hydroxycinnamic acids (e.g. sinapic acid), having the same hydroxyl and methoxy groups as hydroxybenzoates (e.g. syringic acid), are more effective in scavenging radicals. It has been reported that the conjugated double bond in the side chain of hydroxycinnamics contribute to their antioxidant activity by stabilizing radicals (Chen et al., 1997; Foti et al., 1996). Nearly all the phenolic acid standards tested for their radical scavenging activity were more potent antioxidants than Trolox, except for 2-hydroxycinnamic and 3-hydroxycinnamic acids. The average slope of 26.0 determined for Trolox during the current work correlates very well with the 26.5 determined for Trolox by Obón et al. (2005), indicating good reproducibility of the TEAC assay.

The presence of the hydroxybenzoic and hydroxycinnamic acids are confirmed in the three plant extracts (Table 2). Subsequently, the antioxidant activity of the crude methanol and water extracts may be attributable to their content of these compounds. Crude aqueous extracts showed a positive correlation between phenolic content and antioxidant activity with a correlation coefficient of $r = 0.66$, which is in agreement with literature (Velioglu et al., 1998; Shan et al., 2005). However, the correlation coefficient between the total phenolic content and antioxidant activity of crude methanol extracts was determined to be $r =$

0.04, indicating little correlation between phenolic contents and antioxidant activity in crude methanol extracts. This is in contrast to the observations by others who found a high correlation between antioxidant activities of plant materials/ products with their total content of phenolic compounds (Velioglu et al., 1998; Shan et al., 2005). The weak correlation may be explained by the fact that the antioxidant activity of plant materials not only depends on the concentration but also on the structure and synergistic interactions between antioxidants (Djerdane et al., 2006). Moreover, the F-C reagent is affected by several other interfering non-phenolic substances such as organic substances e.g. proteins and sugars in crude plant extracts (Box, 1983). Shan et al. (2005) also reported that it is difficult to obtain good correlations between phenolic content and antioxidant activity when the ranges of the values obtained for total phenolic content and antioxidant activity are very small.

In conclusion, the three medicinal plants investigated were found to be strong radical scavengers supporting the traditional use of these medicinal plants.

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Abbreviations

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **BSTFA**, bis(trimethylsilyl)trifluoroacetamide; **DiIF**, dilution factor; **EI**, electron impact ionization; **FW/DW**, ratio between the fresh and dry plant weight; **GAE**, gallic acid equivalence; **GC**, Gas chromatography; **MS**, Mass spectrometry; **RE**, rutin equivalence; **TEAC**, trolox equivalence antioxidant capacity; **TFC**, total flavonoid content; **TMCS**, trimethylchlorosilane; **TPC**, total phenolic content.

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Full Length Research Paper

The clinical characteristics of anti-tuberculosis drug induced liver injury in 2457 hospitalized patients with tuberculosis in China

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Anti-tuberculosis drug induced liver injury (ATDILI) ranks the first in all kinds of drug-induced liver injuries. ATDILI causes not only economic loss of public health system and patients, but also mental burden of patients, and often leads to treatment interruption or drug resistance. To investigate the clinical characteristics and predisposing factors of ATDILI, the clinical characteristics of 2457 hospital-admitted cases treated with anti-tuberculosis (TB) drugs during 2005 and 2009 and their correlative factors were retrospectively analyzed. The incidence of ATDILI among 2457 TB patients who were treated for the first time was 10.9% (267/2457), that is 13.6% (148/1085) in female and 8.7% (119/1372) in male. Female TB patients were more prone to have ATDILI as compared to male TB patients ($P<0.05$). The incidence of ATDILI in TB patients who were not treated with liver protectants (13.8%, 80/581) was significantly higher than that in patients who were treated with liver protectants (10.0%, 187/1876, $P<0.05$). 13.2% (139/1050) patients treated with isoniazid (H), rifampin (R), and pyrazinamide (Z)/ethambutol (E) (HRZ/E) regime had ATDILI, which was significantly higher than that in TB patients treated with HRE (9.1%, 128/1407, $P<0.05$). Susceptible patients of ATDILI should take appropriate preventive measures to avoid the occurrence of drug-induced liver injury.

Key words: Liver injury, anti-tuberculosis drugs, clinical characteristics, predisposing factor.

INTRODUCTION

It is estimated that 550 million people were infected with *Mycobacterium tuberculosis* in China. The incidence of pulmonary tuberculosis (TB) is 459 per 100,000 population, the incidence of smear-positive or culture-positive pulmonary TB is 66 per 100,000 population (The Ministry of health in China, 2011). The high incidence of TB in China, which is the second highest in the world, only next to India, is accompanied by high incidence of anti-TB drug induced liver injury (ATDILI). Studies have shown that the incidence of liver injury

induced by the multidrug anti-TB regimens is the highest among all drug-induced liver injuries (Zhou et al., 2007; Wang et al., 2009). ATDILI not only leads to delay in chemotherapy, but also affects the control of TB, which consequently seriously endangered the lives of patients. Therefore, the monitoring ATDILI is crucial.

In this study, 2457 hospital-admitted TB cases in China from 2005 to 2009 and the clinical features of 267 ATDILI cases were retrospectively analyzed to understand the general characteristics and the risk factors of ATDILI, and

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to guide prevention and treatment of ATDLI.

MATERIALS AND METHODS

General information

2457 clinically diagnosed TB patients (except those with TB in the central nervous system due to a high dose of isoniazid) who were admitted to Institute of Tuberculosis Research, the 309th Hospital of Chinese PLA, China, from 2005 to 2009 and accepted the chemotherapeutic regimen according to conventional protocol for TB treatment were included in this retrospective analysis.

Anti-TB treatment

Patients were treated for 6 to 9 months as recommended by our national TB program. They were given anti-TB drugs such as isoniazid (H), rifampin (R), and ethambutol (E) or pyrazinamide (Z) daily in the first two or three months and then followed treatment with HR or HRE for 4 to 7 months. The drug dosages were adjusted based on the body weights of patients except for those who were 60 years and above and had viral hepatitis or alcoholic cirrhosis, were given 2/3 of the normal dosage. Owing to drugs, H, R and Z can cause potential liver damage; the patients were divided into two groups: one group was treated with HRZE or HRZ (HRZ/E); the other was treated with HRE.

The liver protectants chosen were not designed beforehand, because this study was retrospective; the use of liver protectants (for example, reduced glutathione, glucuro lactone, or compound glycyrrhizin tablets composed of mono-ammonium glycyrrhizinate, glycine and methionine) was depended on the patients' economic condition or doctors' custom. All patients with liver damage were treated with liver protectants.

Diagnostic and grading criterions for ATDLI

ATDLI was diagnosed according to the Chinese criterion (Xiao et al., 2009) and base on other reports (Turktas et al., 1994; Saukkonen et al., 2006; Aithal et al., 2011), an elevation in the serum concentration of alanine aminotransferase (ALT) and/or total bilirubin (TBIL) exceeding 2 times of the upper normal limit (UNL) was noticed. Then, ATDLI was divided into 3 types as follow: (1) simple liver cell injury, defined as serum ALT level, is more than 2 times of UNL, and alkaline phosphatase (ALP) level is normal but ALT to ALP ratio is greater than or equal to 5; (2) cholestatic liver injury, defined as serum ALP level is more than 2 times of UNL, and ALT level is normal but or ALT to ALP ratio is less than 2; (3) mixed liver injury, defined as both ALT and ALP level is more than 2 times of UNL, and ALT/ALP ratio is between 2 to 5. Except the following liver dysfunction conditions: (1) malnutrition; (2) alcoholic liver disease or habitual drinking; (3) hepatitis B or C infection, liver disease, systemic diseases and/or treatment with non-anti-TB drugs that can induce hepatotoxicity; (4) severe TB or cardiac dysfunction that may cause liver dysfunction; and (5) transient increase in ALT, AST or TBIL, the severity of hepatotoxicity was classified according to the WHO Toxicity Classification Standards (Tostmann et al., 2008; Aithal et al., 2011). ATDLI was classified into 3 grades: (1) mild ATDLI, defined as serum ALT level is 2 to 5 times of UNL and normal TBIL level; (2) moderate ATDLI, defined as serum ALT level is 5 to 10 times of UNL, or serum ALT or AST level is less than 5 times of UNL and TBIL level is 2 to 5 times of UNL; (3) severe ATDLI, defined as both ALT or AST and TBIL level is more than 5 times of UNL, or TBIL

level is more than 2 times of UNL with ascites and/or encephalopathy or other organ failure.

Treatment of patients with ATDLI

Patients with mild ATDLI were continuously treated with anti-TB drugs after treatment with liver protectants. Patients with moderate ATDLI were provided with detoxification, liver protectants and ALT reducing treatment such as orally taking glucuro lactone, liver protectant tablets, intravenously infusing diammonium glycyrrhizinate, reduced glutathione, etc., and not further treated with anti-TB drugs. Patients with severe ATDLI were treated as follow: (1) immediately stopped taking all anti-TB drugs in order to remove the cause of the disease; (2) started taking ursodeoxycholic acid as early as possible for short period to accelerate the jaundice disappearing; (3) intravenously administrated diammonium glycyrrhizinate, S-adenosyl methionine, essential, reduced glutathione, etc., to accelerate liver detoxification and promote liver cell repair; (4) intravenously administered vitamin, branched-chain amino acids and albumin; (5) given other symptomatic treatment.

Data analysis

The correlations of age, gender, history of liver disease and liver protectants and anti-TB regimes with ATDLI were analyzed using χ^2 test with SPSS statistical software package. The difference of ATDLI incidence between the HRZ/E and HRE groups was analyzed statistically in the first two months during the treatment. A P value less than 0.05 was considered statistically significant.

RESULTS

Sample characteristics

Among 2457 TB patients, 1372 cases (55.8%) were male and 1085 cases (44.2%) female. They were between 15 and 95 years old with average of 44.2 ± 15.3 . 897 patients were 60 years and above, accounting for 36.5%, 1560 were younger than 60 years, accounting for 63.5%.

Liver function of all the 2457 TB patients was normal before receiving anti-TB drug treatment. But 358 patients had type B viral hepatitis, fatty liver and alcoholic liver diseases, accounting for 14.6%. 2099 patients had no previous history of liver disease, accounting for 85.4%, among them, 1876 cases (76.4%) were additionally treated with liver protectants and 581 cases (23.6%) were not treated with liver protectants after treatment with anti-TB drugs. Total 267 cases (10.4%) had ATDLI. The injury occurred within the first 2 months of treatment in 201 cases, accounting for 75.2%, of which 28 cases (10.5%) occurred within 2 weeks of treatment. The injury occurred between 2 and 6 months of treatment in 54 cases (20.2%) and occurred after 6 months of treatment in 12 cases (0.4%).

Among these patients, 134 (50.2%) had mild ATDLI, 87 (32.6%) had moderate ATDLI, 46 (17.2%) had severe ATDLI; 184 (68.9%) had simple liver cell injury, 31 (11.6%)

had cholestatic liver injury, and 52 (19.5%) had mixed liver injury.

Clinical manifestations

Among the 267 patients with ATDILI, 14 (5.2%) patients had no obvious clinical manifestations, and 253 (94.8%) had obvious clinical manifestations, in which 246 (92.1%) had fatigue, anorexia, nausea, abdominal distension, diarrhea and other gastrointestinal symptoms; 57 (21.3%) had jaundice; 12 (5%) had hepatomegaly; 3 (1.1%) had hepatic encephalopathy; 187 (76.0%) had malaise; 10 (3.7%) had ascites; 47 (17.6%) had skin rash; and 34 (12.7%) had fever. All the 3 patients with hepatic encephalopathy had severe ATDILI. All patients with severe ATDILI had clinical symptoms.

Blood test results

Among the 267 patients with ATDILI, 33 (12.4%) patients had increased blood eosinophils, in whom 93.9% (31/33) were associated with skin rash; 19 (7.1%) had increased leucocytes; 59 (22.1%) had reduced leucocytes; and 28 (20.7%) had decreased thrombocytes.

Correlation of age with ATDILI

Among the 2457 TB patients, 86 out of 897 (9.6%) patients who were 60 years and above had ATDILI, and 181 out of 1560 (12.0%) patients who were younger than 60 years had ATDILI. The incidence of ATDILI between the two groups was not significantly different ($P>0.05$).

Correlation of gender with ATDILI

Out of 1372 male patients, 119 (9.6%) had ATDILI, whereas 148 out of the 1085 (13.6%) female patients had ATDILI. Compared with male patients, women seem to be more susceptible to ATDILI ($P<0.05$).

Correlation of previous history of liver disease with ATDILI

Among 358 patients who had had hepatitis, fatty liver and other liver diseases, 31 (8.7%) had ATDILI. Among 2099 patients with no history of liver disease, 236 (11.2%) had ATDILI. The incidence of ATDILI was not significantly different between these two groups ($P>0.05$).

Effect of liver protectants on the incidence of ATDILI

Among 1876 patients (76.4%) who were treated with liver

protectants, 187 (10.0%) had ATDILI. Among 581 patients (23.6%) who were not treated with liver protectants, 80 (13.8%) had ATDILI, which was significantly higher than that of patients who received liver protectants ($P<0.05$).

Correlation of anti-TB regimes with ATDILI

Among 1050 cases (42.7%) primarily treated with HRZ/E regimen, 139 (13.2%) had ATDILI, in which 104 (9.9%) occurred within the first two months. Among 1407 cases (57.3%) treated with HRE regimen, 128 (9.1%) had ATDILI, in which 97 (6.9%) occurred within the first two months. The incidence of ATDILI in HRZ/E group increased significantly than that in HRE group in the first two months of treatment ($P<0.05$).

Outcome of treatment

Liver function of all patients with mild to moderate ATDILI was recovered after treatment. Among the 46 patients with severe ATDILI, 42 recovered after being treated with liver protectants, 1 did not recover, 3 died of liver failure. Among these 46 patients, 33 (75.0%) patients had liver cell injury and cholestasis, 8 patients had fever, rash, increased eosinophils and other allergic manifestations. All the 4 patients who died or did not recover after treatment had clinical manifestations of fever and rash. One patient admitted to our Institute 10 days after the onset of fever, rash, and increased blood eosinophils, but not gastrointestinal symptoms, was found having elevated transaminase and bilirubin level, and diagnosed with severe hepatitis; the delay of diagnosis resulted in severe outcome.

DISCUSSION

ATDILI is the most common adverse reactions during the course of regular chemotherapy. It occurred in 0.8 to 34.9% TB patients treated with chemotherapy. The incidence of ATDILI was slightly higher in Asian countries than in Western countries (Gulbay et al., 2006; Sun et al., 2009; Agal et al., 2005; Fernandez et al., 2004; Singanayagam et al., 2012). This study found that 10.4% (267/2457) TB patients had ATDILI, which occurred within 2 months of chemotherapy in 75.2% patients, in consistence with previous reports: Sun et al. (2009) reported that hepatitis occurred in 42 TB patients (16.1%), with 60% of the events in the first 2 months of treatment. Devarbhavi et al. (2013) found that three-quarter ATDILI, and Shang et al. (2011) 71.59% ATDILI occurred within the first 2 months. All of these suggested that the monitoring of liver function was very important in the first two month during the treatment. Among the patients with

ATDILI, 68.9% had liver cell injury, 19.5% had mixed liver injury and 11.6% had cholestasis. The incidence of severe ATDILI is very low, 75.0% of which was mixed ATDILI. Consistent with domestic and overseas researches, this study found that most of ATDILI patients, if found at early stage, could be cured with proper treatment and their liver function could be restored.

This study found that allergic manifestations such as fever (12.7%), rash (17.6%) and increased eosinophils (12.4%) could occur before or accompany at the same time with ATDILI in some patients. Shao et al. (2007) retrospectively analyzed 29 cases with ATDILI and found that up to 11 (37.9%) patients had elevated blood eosinophil percentage. Yin et al. (2008) reported that 2 of 116 cases (1.7%) with ATDILI had fever, joint pain and increased blood eosinophils. These results suggested that allergic factors played an important role in ATDILI. Therefore, when TB patients had allergic manifestations such as fever, rash and increased blood eosinophils, liver function should be examined in time to prevent drug induced-severe liver disease and negative consequences.

Many studies have shown that TB patients with viral hepatitis, history of liver disease, or carrying hepatitis virus were independent factors for development of ATDILI, and the elderly and women were prone to have ATDILI (Sun et al., 2009; Fernandez et al., 2004; Shakya et al., 2004; Wong et al., 2000). Col et al. (2006) has reported that patients with hepatitis B virus (HBV) infection had significantly higher incidence of ATDILI (37.5%, 9/24) than those without HBV infection (10.2%, 13/128, $P < 0.01$). In China, due to high HBV infection, TB patient concurred with chronic hepatitis B is common in clinic. The study from Guo et al. (2005) showed that among 132 TB cases with HBV infection in China, the incidence of ATDILI was 35.61%, significantly higher than those of 17.02% in 94 TB patients without HBV infection. Yin et al. (2008) found that TB patients with positive hepatitis B surface antigen (HBsAg) and older than 60 years old had higher incidence of ATDILI, which was 32.1% (18/56) and 22.9% (8/35), respectively, compared with that of TB patients with negative HBsAg or younger than 60 years old ($P < 0.05$). Fernández et al. (2004) found in a retrospective study that 56 out of 471 TB patients had clinical features of ATDILI, among them, elder TB patients and TB patients with liver disease had increased incidence of ATDILI ($P < 0.001$). All of the aforementioned researches have prompted that the elderly and TB patients with liver disease are the high-risk population of ATDILI. The reasons that the elderly are more vulnerable to ATDILI may be weakened drug biotransformation and excretion resulting from less liver blood flow, reduced liver cell function, and decreased liver microsomal enzyme amount and activity. TB patients with liver disease are prone to have ATDILI, which is because they already had liver damage before application of anti-TB drugs. These results are in contrast to a previous report of Gulbay et al. (2006) that retrospective evaluation

of 1149 TB patients who initially received anti-TB therapy did not observe age differences in patients with and without hepatotoxicity. The current study did not find TB patients with history of viral hepatitis and other liver diseases and elderly patients are prone to have ATDILI, which may be related to the following factors: (1) decreased anti-TB drug dosage had been clinically taken into account in these patients; (2) application of liver protection drugs reduced the incidence of ATDILI; (3) the liver injury was induced by many factors. It was also found out that TB patients without liver protectant had significantly higher ATDILI incidence (13.8%, 80/581) than that of TB patients with liver protectant (10.0%, 187/1876, $P < 0.05$). These results further suggested that susceptible patients of ATDILI should take appropriate preventive measures to avoid the occurrence of drug-induced liver injury. Because this study was retrospective, the liver protectants chosen were not designed beforehand, and the use of liver protectants was depended on the patients' economic condition or doctors' custom. Therefore, we could not further analyze the effect of different liver protectants, which is worthy of further study in the future.

This study found that the incidence of ATDILI was higher in female patients than that in male patients, which is similar to a previous report by Hunt et al. (1992). They proposed that higher incidence of liver damage induced by some non-anti-TB drugs in female may be related to higher CYP3A activity (Hunt et al., 1992), but the relationship of CYP3A activity with ATDILI is unclear. Col et al. (2006) showed that among 69 TB patients with ATDILI and 70 TB patients without ATDILI, the proportion between male and female patients was not significantly different ($P > 0.05$). Similarly, Gulbay et al. (2006) also did not observe gender differences in 1149 TB patients with and without hepatotoxicity. However, Shao et al. (2007) reported a contrary result among 29 ATDILI cases, 20 (68.97%) were male and 9 (31.03%) were female. These differences may be associated with case selection and sample number. The relationship between gender and ATDILI requires further study.

The study also found that the incidence of ATDILI in TB patients treated with HRZ/E regimen was significantly higher than that in TB patients treated with HRE regimen ($P < 0.05$), which is consistent with the report by Hang et al. (2008) that the incidence of ATDILI was 2.6% significantly higher in patients treated with HRZ/E regimen than that of 0.8% in patients treated with HRE regimen ($P < 0.05$). This result indicates that HRZ/E regimen is more toxic to liver than HRE, possibly related to the toxicity accumulation of higher liver toxic pyrazinamide, or the increased liver toxicity of isoniazid and/or rifampin that resulted from the interference of pyrazinamide with the metabolism of isoniazid and/or rifampin. Therefore, TB patients with high ATDILI risk should select treatment regimen strictly according to the condition of individuals.

This study was retrospective. Therefore, there were some

limitations: first, some laboratory variables were missed, which hindered us from studying the relationship between serum albumin and outcome of ATDILI (Devarbhavi et al., 2013); second, owing to the fact that the treatment regimes were not designed in advance, some patients received the treatment with regimes HRZ/E, some patients with regimes HRZ, which might affect the incidence of ATDILI. But we could know the difference of ATDILI incidence between the two regimes.

Conclusively, ATDILI is a common complication during anti-TB chemotherapy, which causes not only economic loss of public health system and patients, but also mental burden of patients, and often leads to treatment interruption or drug resistance. Further study on the molecular mechanism of ATDILI and clinical predisposing factors is vital. For population susceptible to ATDILI, selective anti-TB chemotherapy should be applied for individuals to reduce the incidence of ATDILI.

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Full Length Research Paper

The clinical characteristics of anti-tuberculosis drug induced liver injury in 2457 hospitalized patients with tuberculosis in China

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Anti-tuberculosis drug induced liver injury (ATDILI) ranks the first in all kinds of drug-induced liver injuries. ATDILI causes not only economic loss of public health system and patients, but also mental burden of patients, and often leads to treatment interruption or drug resistance. To investigate the clinical characteristics and predisposing factors of ATDILI, the clinical characteristics of 2457 hospital-admitted cases treated with anti-tuberculosis (TB) drugs during 2005 and 2009 and their correlative factors were retrospectively analyzed. The incidence of ATDILI among 2457 TB patients who were treated for the first time was 10.9% (267/2457), that is 13.6% (148/1085) in female and 8.7% (119/1372) in male. Female TB patients were more prone to have ATDILI as compared to male TB patients ($P<0.05$). The incidence of ATDILI in TB patients who were not treated with liver protectants (13.8%, 80/581) was significantly higher than that in patients who were treated with liver protectants (10.0%, 187/1876, $P<0.05$). 13.2% (139/1050) patients treated with isoniazid (H), rifampin (R), and pyrazinamide (Z)/ethambutol (E) (HRZ/E) regime had ATDILI, which was significantly higher than that in TB patients treated with HRE (9.1%, 128/1407, $P<0.05$). Susceptible patients of ATDILI should take appropriate preventive measures to avoid the occurrence of drug-induced liver injury.

Key words: Liver injury, anti-tuberculosis drugs, clinical characteristics, predisposing factor.

INTRODUCTION

It is estimated that 550 million people were infected with *Mycobacterium tuberculosis* in China. The incidence of pulmonary tuberculosis (TB) is 459 per 100,000 population, the incidence of smear-positive or culture-positive pulmonary TB is 66 per 100,000 population (The Ministry of health in China, 2011). The high incidence of TB in China, which is the second highest in the world, only next to India, is accompanied by high incidence of anti-TB drug induced liver injury (ATDILI). Studies have shown that the incidence of liver injury

induced by the multidrug anti-TB regimens is the highest among all drug-induced liver injuries (Zhou et al., 2007; Wang et al., 2009). ATDILI not only leads to delay in chemotherapy, but also affects the control of TB, which consequently seriously endangered the lives of patients. Therefore, the monitoring ATDILI is crucial.

In this study, 2457 hospital-admitted TB cases in China from 2005 to 2009 and the clinical features of 267 ATDILI cases were retrospectively analyzed to understand the general characteristics and the risk factors of ATDILI, and

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to guide prevention and treatment of ATDLI.

MATERIALS AND METHODS

General information

2457 clinically diagnosed TB patients (except those with TB in the central nervous system due to a high dose of isoniazid) who were admitted to Institute of Tuberculosis Research, the 309th Hospital of Chinese PLA, China, from 2005 to 2009 and accepted the chemotherapeutic regimen according to conventional protocol for TB treatment were included in this retrospective analysis.

Anti-TB treatment

Patients were treated for 6 to 9 months as recommended by our national TB program. They were given anti-TB drugs such as isoniazid (H), rifampin (R), and ethambutol (E) or pyrazinamide (Z) daily in the first two or three months and then followed treatment with HR or HRE for 4 to 7 months. The drug dosages were adjusted based on the body weights of patients except for those who were 60 years and above and had viral hepatitis or alcoholic cirrhosis, were given 2/3 of the normal dosage. Owing to drugs, H, R and Z can cause potential liver damage; the patients were divided into two groups: one group was treated with HRZE or HRZ (HRZ/E); the other was treated with HRE.

The liver protectants chosen were not designed beforehand, because this study was retrospective; the use of liver protectants (for example, reduced glutathione, glucuro lactone, or compound glycyrrhizin tablets composed of mono-ammonium glycyrrhizinate, glycine and methionine) was depended on the patients' economic condition or doctors' custom. All patients with liver damage were treated with liver protectants.

Diagnostic and grading criterions for ATDLI

ATDLI was diagnosed according to the Chinese criterion (Xiao et al., 2009) and base on other reports (Turktas et al., 1994; Saukkonen et al., 2006; Aithal et al., 2011), an elevation in the serum concentration of alanine aminotransferase (ALT) and/or total bilirubin (TBIL) exceeding 2 times of the upper normal limit (UNL) was noticed. Then, ATDLI was divided into 3 types as follow: (1) simple liver cell injury, defined as serum ALT level, is more than 2 times of UNL, and alkaline phosphatase (ALP) level is normal but ALT to ALP ratio is greater than or equal to 5; (2) cholestatic liver injury, defined as serum ALP level is more than 2 times of UNL, and ALT level is normal but or ALT to ALP ratio is less than 2; (3) mixed liver injury, defined as both ALT and ALP level is more than 2 times of UNL, and ALT/ALP ratio is between 2 to 5. Except the following liver dysfunction conditions: (1) malnutrition; (2) alcoholic liver disease or habitual drinking; (3) hepatitis B or C infection, liver disease, systemic diseases and/or treatment with non-anti-TB drugs that can induce hepatotoxicity; (4) severe TB or cardiac dysfunction that may cause liver dysfunction; and (5) transient increase in ALT, AST or TBIL, the severity of hepatotoxicity was classified according to the WHO Toxicity Classification Standards (Tostmann et al., 2008; Aithal et al., 2011). ATDLI was classified into 3 grades: (1) mild ATDLI, defined as serum ALT level is 2 to 5 times of UNL and normal TBIL level; (2) moderate ATDLI, defined as serum ALT level is 5 to 10 times of UNL, or serum ALT or AST level is less than 5 times of UNL and TBIL level is 2 to 5 times of UNL; (3) severe ATDLI, defined as both ALT or AST and TBIL level is more than 5 times of UNL, or TBIL

level is more than 2 times of UNL with ascites and/or encephalopathy or other organ failure.

Treatment of patients with ATDLI

Patients with mild ATDLI were continuously treated with anti-TB drugs after treatment with liver protectants. Patients with moderate ATDLI were provided with detoxification, liver protectants and ALT reducing treatment such as orally taking glucuro lactone, liver protectant tablets, intravenously infusing diammonium glycyrrhizinate, reduced glutathione, etc., and not further treated with anti-TB drugs. Patients with severe ATDLI were treated as follow: (1) immediately stopped taking all anti-TB drugs in order to remove the cause of the disease; (2) started taking ursodeoxycholic acid as early as possible for short period to accelerate the jaundice disappearing; (3) intravenously administrated diammonium glycyrrhizinate, S-adenosyl methionine, essential, reduced glutathione, etc., to accelerate liver detoxification and promote liver cell repair; (4) intravenously administered vitamin, branched-chain amino acids and albumin; (5) given other symptomatic treatment.

Data analysis

The correlations of age, gender, history of liver disease and liver protectants and anti-TB regimes with ATDLI were analyzed using χ^2 test with SPSS statistical software package. The difference of ATDLI incidence between the HRZ/E and HRE groups was analyzed statistically in the first two months during the treatment. A P value less than 0.05 was considered statistically significant.

RESULTS

Sample characteristics

Among 2457 TB patients, 1372 cases (55.8%) were male and 1085 cases (44.2%) female. They were between 15 and 95 years old with average of 44.2 ± 15.3 . 897 patients were 60 years and above, accounting for 36.5%, 1560 were younger than 60 years, accounting for 63.5%.

Liver function of all the 2457 TB patients was normal before receiving anti-TB drug treatment. But 358 patients had type B viral hepatitis, fatty liver and alcoholic liver diseases, accounting for 14.6%. 2099 patients had no previous history of liver disease, accounting for 85.4%, among them, 1876 cases (76.4%) were additionally treated with liver protectants and 581 cases (23.6%) were not treated with liver protectants after treatment with anti-TB drugs. Total 267 cases (10.4%) had ATDLI. The injury occurred within the first 2 months of treatment in 201 cases, accounting for 75.2%, of which 28 cases (10.5%) occurred within 2 weeks of treatment. The injury occurred between 2 and 6 months of treatment in 54 cases (20.2%) and occurred after 6 months of treatment in 12 cases (0.4%).

Among these patients, 134 (50.2%) had mild ATDLI, 87 (32.6%) had moderate ATDLI, 46 (17.2%) had severe ATDLI; 184 (68.9%) had simple liver cell injury, 31 (11.6%)

had cholestatic liver injury, and 52 (19.5%) had mixed liver injury.

Clinical manifestations

Among the 267 patients with ATDILI, 14 (5.2%) patients had no obvious clinical manifestations, and 253 (94.8%) had obvious clinical manifestations, in which 246 (92.1%) had fatigue, anorexia, nausea, abdominal distension, diarrhea and other gastrointestinal symptoms; 57 (21.3%) had jaundice; 12 (5%) had hepatomegaly; 3 (1.1%) had hepatic encephalopathy; 187 (76.0%) had malaise; 10 (3.7%) had ascites; 47 (17.6%) had skin rash; and 34 (12.7%) had fever. All the 3 patients with hepatic encephalopathy had severe ATDILI. All patients with severe ATDILI had clinical symptoms.

Blood test results

Among the 267 patients with ATDILI, 33 (12.4%) patients had increased blood eosinophils, in whom 93.9% (31/33) were associated with skin rash; 19 (7.1%) had increased leucocytes; 59 (22.1%) had reduced leucocytes; and 28 (20.7%) had decreased thrombocytes.

Correlation of age with ATDILI

Among the 2457 TB patients, 86 out of 897 (9.6%) patients who were 60 years and above had ATDILI, and 181 out of 1560 (12.0%) patients who were younger than 60 years had ATDILI. The incidence of ATDILI between the two groups was not significantly different ($P>0.05$).

Correlation of gender with ATDILI

Out of 1372 male patients, 119 (9.6%) had ATDILI, whereas 148 out of the 1085 (13.6%) female patients had ATDILI. Compared with male patients, women seem to be more susceptible to ATDILI ($P<0.05$).

Correlation of previous history of liver disease with ATDILI

Among 358 patients who had had hepatitis, fatty liver and other liver diseases, 31 (8.7%) had ATDILI. Among 2099 patients with no history of liver disease, 236 (11.2%) had ATDILI. The incidence of ATDILI was not significantly different between these two groups ($P>0.05$).

Effect of liver protectants on the incidence of ATDILI

Among 1876 patients (76.4%) who were treated with liver

protectants, 187 (10.0%) had ATDILI. Among 581 patients (23.6%) who were not treated with liver protectants, 80 (13.8%) had ATDILI, which was significantly higher than that of patients who received liver protectants ($P<0.05$).

Correlation of anti-TB regimes with ATDILI

Among 1050 cases (42.7%) primarily treated with HRZ/E regimen, 139 (13.2%) had ATDILI, in which 104 (9.9%) occurred within the first two months. Among 1407 cases (57.3%) treated with HRE regimen, 128 (9.1%) had ATDILI, in which 97 (6.9%) occurred within the first two months. The incidence of ATDILI in HRZ/E group increased significantly than that in HRE group in the first two months of treatment ($P<0.05$).

Outcome of treatment

Liver function of all patients with mild to moderate ATDILI was recovered after treatment. Among the 46 patients with severe ATDILI, 42 recovered after being treated with liver protectants, 1 did not recover, 3 died of liver failure. Among these 46 patients, 33 (75.0%) patients had liver cell injury and cholestasis, 8 patients had fever, rash, increased eosinophils and other allergic manifestations. All the 4 patients who died or did not recover after treatment had clinical manifestations of fever and rash. One patient admitted to our Institute 10 days after the onset of fever, rash, and increased blood eosinophils, but not gastrointestinal symptoms, was found having elevated transaminase and bilirubin level, and diagnosed with severe hepatitis; the delay of diagnosis resulted in severe outcome.

DISCUSSION

ATDILI is the most common adverse reactions during the course of regular chemotherapy. It occurred in 0.8 to 34.9% TB patients treated with chemotherapy. The incidence of ATDILI was slightly higher in Asian countries than in Western countries (Gulbay et al., 2006; Sun et al., 2009; Agal et al., 2005; Fernandez et al., 2004; Singanayagam et al., 2012). This study found that 10.4% (267/2457) TB patients had ATDILI, which occurred within 2 months of chemotherapy in 75.2% patients, in consistence with previous reports: Sun et al. (2009) reported that hepatitis occurred in 42 TB patients (16.1%), with 60% of the events in the first 2 months of treatment. Devarbhavi et al. (2013) found that three-quarter ATDILI, and Shang et al. (2011) 71.59% ATDILI occurred within the first 2 months. All of these suggested that the monitoring of liver function was very important in the first two month during the treatment. Among the patients with

ATDILI, 68.9% had liver cell injury, 19.5% had mixed liver injury and 11.6% had cholestasis. The incidence of severe ATDILI is very low, 75.0% of which was mixed ATDILI. Consistent with domestic and overseas researches, this study found that most of ATDILI patients, if found at early stage, could be cured with proper treatment and their liver function could be restored.

This study found that allergic manifestations such as fever (12.7%), rash (17.6%) and increased eosinophils (12.4%) could occur before or accompany at the same time with ATDILI in some patients. Shao et al. (2007) retrospectively analyzed 29 cases with ATDILI and found that up to 11 (37.9%) patients had elevated blood eosinophil percentage. Yin et al. (2008) reported that 2 of 116 cases (1.7%) with ATDILI had fever, joint pain and increased blood eosinophils. These results suggested that allergic factors played an important role in ATDILI. Therefore, when TB patients had allergic manifestations such as fever, rash and increased blood eosinophils, liver function should be examined in time to prevent drug induced-severe liver disease and negative consequences.

Many studies have shown that TB patients with viral hepatitis, history of liver disease, or carrying hepatitis virus were independent factors for development of ATDILI, and the elderly and women were prone to have ATDILI (Sun et al., 2009; Fernandez et al., 2004; Shakya et al., 2004; Wong et al., 2000). Col et al. (2006) has reported that patients with hepatitis B virus (HBV) infection had significantly higher incidence of ATDILI (37.5%, 9/24) than those without HBV infection (10.2%, 13/128, $P < 0.01$). In China, due to high HBV infection, TB patient concurred with chronic hepatitis B is common in clinic. The study from Guo et al. (2005) showed that among 132 TB cases with HBV infection in China, the incidence of ATDILI was 35.61%, significantly higher than those of 17.02% in 94 TB patients without HBV infection. Yin et al. (2008) found that TB patients with positive hepatitis B surface antigen (HBsAg) and older than 60 years old had higher incidence of ATDILI, which was 32.1% (18/56) and 22.9% (8/35), respectively, compared with that of TB patients with negative HBsAg or younger than 60 years old ($P < 0.05$). Fernández et al. (2004) found in a retrospective study that 56 out of 471 TB patients had clinical features of ATDILI, among them, elder TB patients and TB patients with liver disease had increased incidence of ATDILI ($P < 0.001$). All of the aforementioned researches have prompted that the elderly and TB patients with liver disease are the high-risk population of ATDILI. The reasons that the elderly are more vulnerable to ATDILI may be weakened drug biotransformation and excretion resulting from less liver blood flow, reduced liver cell function, and decreased liver microsomal enzyme amount and activity. TB patients with liver disease are prone to have ATDILI, which is because they already had liver damage before application of anti-TB drugs. These results are in contrast to a previous report of Gulbay et al. (2006) that retrospective evaluation

of 1149 TB patients who initially received anti-TB therapy did not observe age differences in patients with and without hepatotoxicity. The current study did not find TB patients with history of viral hepatitis and other liver diseases and elderly patients are prone to have ATDILI, which may be related to the following factors: (1) decreased anti-TB drug dosage had been clinically taken into account in these patients; (2) application of liver protection drugs reduced the incidence of ATDILI; (3) the liver injury was induced by many factors. It was also found out that TB patients without liver protectant had significantly higher ATDILI incidence (13.8%, 80/581) than that of TB patients with liver protectant (10.0%, 187/1876, $P < 0.05$). These results further suggested that susceptible patients of ATDILI should take appropriate preventive measures to avoid the occurrence of drug-induced liver injury. Because this study was retrospective, the liver protectants chosen were not designed beforehand, and the use of liver protectants was depended on the patients' economic condition or doctors' custom. Therefore, we could not further analyze the effect of different liver protectants, which is worthy of further study in the future.

This study found that the incidence of ATDILI was higher in female patients than that in male patients, which is similar to a previous report by Hunt et al. (1992). They proposed that higher incidence of liver damage induced by some non-anti-TB drugs in female may be related to higher CYP3A activity (Hunt et al., 1992), but the relationship of CYP3A activity with ATDILI is unclear. Col et al. (2006) showed that among 69 TB patients with ATDILI and 70 TB patients without ATDILI, the proportion between male and female patients was not significantly different ($P > 0.05$). Similarly, Gulbay et al. (2006) also did not observe gender differences in 1149 TB patients with and without hepatotoxicity. However, Shao et al. (2007) reported a contrary result among 29 ATDILI cases, 20 (68.97%) were male and 9 (31.03%) were female. These differences may be associated with case selection and sample number. The relationship between gender and ATDILI requires further study.

The study also found that the incidence of ATDILI in TB patients treated with HRZ/E regimen was significantly higher than that in TB patients treated with HRE regimen ($P < 0.05$), which is consistent with the report by Hang et al. (2008) that the incidence of ATDILI was 2.6% significantly higher in patients treated with HRZ/E regimen than that of 0.8% in patients treated with HRE regimen ($P < 0.05$). This result indicates that HRZ/E regimen is more toxic to liver than HRE, possibly related to the toxicity accumulation of higher liver toxic pyrazinamide, or the increased liver toxicity of isoniazid and/or rifampin that resulted from the interference of pyrazinamide with the metabolism of isoniazid and/or rifampin. Therefore, TB patients with high ATDILI risk should select treatment regimen strictly according to the condition of individuals.

This study was retrospective. Therefore, there were some

limitations: first, some laboratory variables were missed, which hindered us from studying the relationship between serum albumin and outcome of ATDILI (Devarbhavi et al., 2013); second, owing to the fact that the treatment regimes were not designed in advance, some patients received the treatment with regimes HRZ/E, some patients with regimes HRZ, which might affect the incidence of ATDILI. But we could know the difference of ATDILI incidence between the two regimes.

Conclusively, ATDILI is a common complication during anti-TB chemotherapy, which causes not only economic loss of public health system and patients, but also mental burden of patients, and often leads to treatment interruption or drug resistance. Further study on the molecular mechanism of ATDILI and clinical predisposing factors is vital. For population susceptible to ATDILI, selective anti-TB chemotherapy should be applied for individuals to reduce the incidence of ATDILI.

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Full Length Research Paper

Study on mock strawberry extracts *in-vitro* anti-tumor activity

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This study was aimed at investigating the anti-tumor effects of mock strawberry extracts on S180 tumor-bearing mice and exploring its action mechanism. Solid tumor mice models were made and drugs were administered based of grouping. The tumor bodies were used to measure the anti-tumor rate, the blood was used to measure the interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α) contents in the serum; while the thymus and spleen were obtained to measure their respective indexes. The mock strawberry extracts can inhibit tumor to some extent, increase the contents of IL-2 and TNF- α in the serum and improve the thymus and spleen indexes in some measure. Mock strawberry extracts may act by improving the immunity of the organism to achieve *in-vitro* anti-tumor activity.

Key words: Mock strawberry, S180 sarcoma, IL-2; interleukin-2 (IL-2), tumor necrosis factor- α (TNF- α), immunity.

INTRODUCTION

First written in Miscellaneous Records of Famous Physicians—a famous medical science book of ancient China, and nicknamed cockscomb berry, myrica rubra, arbutus etc., mock strawberry is the whole grass for Rosaceae plant - *Duchesnea indica* (Andr.) Focke, used for curing febrile disease, cough, hematemesis, swollen sore throat and eczema (New Jiangsu Medical School, 1997; Hong-wei, 2002). The chemical compositions of mock strawberry are triterpene and glycosides, coumarins, flavonoid, sterols etc.

It has been reported that mock strawberry water extract has cytotoxicity effect on human hepatoma (7721),

gastric cancer (7901) and esophageal cancer (Eca109) (Jing-yun et al., 1998). Mock strawberry total phenol inhibits proliferating tumor cells in a dose-dependence manner, so it acts obviously in *in-vitro* anti-tumor (Bo et al., 2007). Mock strawberry aqueous extracts act well in inhibiting the growth of lung cancer (LLC), pancreatic cancer (Panc02) and mammary cancer (MC-NeuA) cells of the mice, with IC₅₀ of 217, 206 and 311 μ g/ml, respectively (Shoemaker et al., 2005). Sarcoma 180 (S180) solid tumor animal model was adopted in this paper to study the anti-tumor mechanism of mock strawberry extracts.

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MATERIALS AND METHODS

Drug and reagent

Cyclophosphamide was purchased from Jiang Su Heng Rui Co. Ltd., while IL-2 and TNF radio immunoassay kits were purchased from Beijing Furui Biological Technology Co., Ltd., Beijing.

Experimental animal and S180 Tumor strain

Male and female Kunming mice, each weighing 18 to 22g, were purchased from the Experimental Animal Centre of Harbin Medical University, with license no. 2011-8-56. The S180 tumor strain was purchased from Nanjing KeyGen Biotech. Inc., Nanjing, China.

Preparation of mock strawberry extract

To obtain mock strawberry extract, appropriate amount of mock strawberry materials were crushed and 60% ethanol was added. Three 30 mm ultrasonic extraction was conducted and combine the extracting solutions. After concentrating in low temperature, polyamide resins was purified, and the mock strawberry extract powders (polyphenols) were obtained after the outflow liquid was concentrated in low temperature and dried. When needed, these were prepared into solutions of proper density for use.

Cell culture

Cells were prepared into single cell suspension, and were adjusted to 1×10^6 . 0.2 ml of the suspension was transplanted into the peritoneal cavity of each mouse under the conditions of asepsis, and was observed for the expansion of the abdomen every day. After seven days, seroperitoneum of the mice were extracted.

Model building

After seven days of vaccination, the mice were executed by cervical dislocation when their abdominal circumference increases to the largest. After disinfecting their abdomen, they were then cut-off and the seroperitoneum was extracted with 1 ml sterilized syringe. The seroperitoneum was diluted with PBS solution, centrifuged (10 min) at 1000 rpm/min to give up the supernatant, and the cells were counted with trypan blue dye and the number of cells was adjusted to 1×10^6 ml⁻¹. In a circle of 50 mice, their subaxillary cutaneous was disinfected and infected with 0.2 ml tumor cell suspension into the right forelimb subaxillary with 1 ml sterilized syringe making them into solid tumor models (Yi-kui et al., 2006).

Grouping and processing

After 24 h of vaccination, the mice were classified randomly into model group, cyclophosphamide group, and group with high, medium and low dose of mock strawberry extract; all in 5 groups with ten mice in each group). All the mice were supplied with enough water and food, and were weighed and the record taken. The work of Jing-yun et al. (1998) and the methods in the methodology of pharmacological experiment by Yi-Kui et al. (2006) were used to determine the administration and dose of the drugs, for high, medium, and low doses of mockery extract to be 13.5, 9.0 and 4.5 g/kg, respectively. The mental states, activities and eating of the mice were observed daily. At the 11th day after drug administration, blood was extracted from the eyeball after weighing and serum was extracted after centrifugation. IL-2 and TNF- α

contents in the serum were detected with radioimmunoassay. The mice were executed after extracting the blood, exfoliating the tumor bodies, weighing them and calculating the tumor inhibition rates according to the following formula:

$$\text{Tumor inhibition rate} = (\text{average tumor weight of the control group} - \text{average tumor weight of the experiment group}) / \text{average tumor weight of the control group} \times 100\%$$

The thymus and spleen of each mouse were removed and weighed. The weight (mg) of the thymus and spleen divided by the weight (10 g) of the mice was taken as thymus and spleen indexes.

Statistical methods

The experimental data were analyzed by Statistical Package for Social Sciences (SPSS)13.0 software. Comparison between two groups was done using t-detection and the comparison between many groups was done using one-way analysis of variance (ANOVA).

RESULTS

Effects of mock strawberry extracts on the weight of the mice

The weight changes before and after the experiments are shown in Table 1. The weight of the experimental groups of mice increased obviously except that of the Cyclophosphamide group, but there was small comparative increase for the model group, in which the increase and the dose form a negative correlation.

Effects of mock strawberry extracts on tumor weight and anti-tumor rate

Effects of mock strawberry extracts on tumor weight and anti-tumor rate are shown in Table 2. Compared with the weight of model group, the tumor weight of group with high dose of mock strawberry extracts was significantly different ($P < 0.01$), and the tumor weight of group with medium dose of mock strawberry extracts is also obviously different ($P < 0.01$). The anti-tumor rates of both groups were more than 30%, indicating that the mock strawberry extracts have apparent inhibition on S180 tumor-bearing mice solid tumor. Compared with the model Group, the group with high dose of mock strawberry extracts had micro smaller weight.

Effects of mock strawberry extracts on IL-2 and TNF- α contents in mice serum

Effects of mock strawberry extracts on IL-2 and TNF- α contents in mice serum are shown in Table 3. Compared with that of model group, the IL-2 level of Cyclophosphamide group was lower ($P < 0.01$), and that of TNF- α was higher but not obviously. IL-2 and TNF- α

Table 1. Effects of mock strawberry extracts on the weight of s180 tumor-bearing mice before and after experiment (mean \pm SD, n=10).

Group	Dose (g /Kg)	No. of animals	Average weight of the mice before experiment (g)	Average weight of the mice after experiment (g)
Model group	-	10	22.98 \pm 2.51	30.46 \pm 4.23
Cyclophosphamide group (g)	0.02	10	22.42 \pm 2.78	25.24 \pm 3.77
Group with high dose of mock strawberry extracts	13.5	10	22.74 \pm 2.39	28.69 \pm 4.45
Group with medium dose of mock strawberry extracts	9.0	10	22.36 \pm 2.88	29.88 \pm 4.75
Group with low dose of mock strawberry extracts	4.5	10	22.53 \pm 2.13	30.23 \pm 5.12

Table 2. Effects of mock strawberry extracts on tumor weight and anti-tumor rate.

Group	Dose(g /Kg)	No. of animals	Tumor Weight (g)	Anti-tumor rate (%)
Model group	-	10	2.12 \pm 0.48	-
Cyclophosphamide group (g)	0.02	10	0.78 \pm 0.35**	63.21
Group with high dose of mock strawberry extracts	13.5	10	1.13 \pm 0.39**	46.70
Group with medium dose of mock strawberry extracts	9.0	10	1.35 \pm 0.34*	36.32
Group with low dose of mock strawberry extracts	4.5	10	1.85 \pm 0.44	12.73

Compared with the Model Group, * P<0.05; ** P<0.01

Table 3. Effects of mock strawberry extracts on il-2 and tnf-a contents in mice serum (X \pm S)

Group	Dose(g /Kg)	No. of animals	IL-2 (ng/mL)	TNF-a (ng/mL)
Model group	-	10	12.412 \pm 4.523	0.518 \pm 0.083
Cyclophosphamide group	0.02	10	9.563 \pm 1.554**	0.597 \pm 0.071
Group with high dose of mock strawberry extracts	13.50	10	16.216 \pm 3.158**	0.746 \pm 0.098**
Group with medium dose of mock strawberry extracts	9.00	10	15.492 \pm 2.984*	0.709 \pm 0.114**
Group with low dose of mock strawberry extracts	4.50	10	14.516 \pm 2.135	0.664 \pm 0.089*

Compared with the Model Group, * P<0.05; ** P<0.01

contents of the group with high dose of mock strawberry extracts rise tremendously (P<0.01), those of Group with Medium Dose of Mock Strawberry Extracts also rise obviously, and IL-2 of Group with Low dose of Mock Strawberry Extracts rises little. We can find from this that mock strawberry extracts can add the contents of IL-2 and TNF-a, thereby improving the lethality of organism to tumor cells and inhibiting tumor.

Effects of mock strawberry extracts on the thymus index and spleen index of the S180 tumor-bearing mice immune organs

Effects of mock strawberry extracts on the thymus index and spleen index of the S180 tumor-bearing mice immune

organs are shown in Table 4. Compared with Model Group, the thymus and spleen weights of Cyclophosphamide Group decrease, and the thymus and spleen indexes lower, while the thymus and spleen indexes of the groups of mock strawberry extracts rise, indicating that the extracts can improve the immunity of organisms, enhancing the inhibition on tumor cells.

As an anti-tumor drug, Cyclophosphamide has the side effects of decreasing organism leukocytes and deteriorating immune functions (Bo-qi and Yunlan, 1996). With the advantages of few side effects and being acceptable to patients, Chinese medicine is focused by researchers home and abroad in the tumor researching field. Many researchers have expanded their research from the activities of anti-tumor to the level of anti-tumor action mechanism (Xiao and Rui-xin, 2011). The occurrence and

Table 4. Effects of mock strawberry extracts on the thymus index and spleen index of the s180 tumor-bearing mice immune organs.

Group	Thymus Index (mg/10g mouse weight)	Spleen Index (mg/10g mouse weight)
Model group	19.5±3.8	54.6±5.2
Cyclophosphamide group	17.3±4.5	53.4±6.3
Group with high dose of mock strawberry extracts	24.5±5.3	64.3±5.6
Group with medium dose of mock strawberry extracts	22.1±4.3	60.8±6.3
Group with low dose of mock strawberry extracts	20.2±3.1	58.1±5.7

and development of tumor are closely related to the deteriorating immunity of the whole organism. If the immunity of human can be improved by certain drugs, there will be positive effects on curing tumors. Currently, many drugs have been found to be able to improve human immunity (Xiao-Ming et al., 2009; Hai-Zhen et al., 2008; Jing-Tao, 2004). Inhibiting tumor development and shrinking tumor bodies is a key indicator to judge whether the anti-tumor drugs act on curing tumor. It has been proved in the paper that mock strawberry extracts can inhibit tumor of S180 tumor-bearing mice, and on the basis of *in-vitro* anti-tumor, effects on immunity of organism are further discussed. Moreover, the effects of mock strawberry extracts on improving organism immunity are primarily proved, of which the exact action mechanism is to be further discussed.

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Full Length Research Paper

Antibacterial activities, chemical constitutes and acute toxicity of Egyptian *Origanum majorana* L., *Peganum harmala* L. and *Salvia officinalis* L. essential oils

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Natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases. *Origanum majorana* L., *Peganum harmala* L. and *Salvia officinalis* L. oils had wide range uses as traditional medicinal plants in Egypt. The current study was designed to evaluate the antibacterial activity of *O. majorana*, *P. harmala* and *S. officinalis* essential oils growing in Egypt for first time. The chemical constitutes and toxicity of these oils were also determined to obtain further information on the correlation between the chemical contents and antibacterial activity. The antibacterial effect of the essential oils of *O. majorana*, *P. harmala* and *S. officinalis* oils were studied against some food borne pathogenic bacteria species. The oils of each plant were subjected to gas chromatography-mass spectrometry (GC/MS). The impact of oils administration on the change in rate of weight gain and complete blood picture in hamsters were investigated. *P. harmala* oil had strong antibacterial effect against bacterial species especially at minimum inhibitory concentration (MIC) less than 75.0 µg/ml. From the oil of *P. harmala*, forty one compounds were identified, and the major constituent was 1-hexyl-2-nitrocyclohexane (9.07%). Acute toxicity test was performed on hamsters and showed complete survival after 14 days, and there no toxicity symptoms occurred. This study demonstrated that these essential oils seemed to be destitute of toxic effect which could compromise the medicinal use of these plants in folk medicine.

Keywords: Analysis mass spectrometry, antibacterial activities, acute toxicity, chemical constitutes, gas chromatography, weight gain, *Origanum majorana*, *Peganum harmala*, *Salvia officinalis*.

INTRODUCTION

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. Another big concern is the development of resistance to the antibiotics in current

clinical use. Traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries, and moreover, the use of herbal remedies has risen in the developed countries in the last decade. In this manner, plants continue to be a rich source of therapeutic agents.

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It is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of bacterial infections (Balandrin et al., 1985; Parekh and Chands, 2007b, c)

Gram-positive cocci, particularly *Staphylococcus* species, are predominant among the organisms that are responsible for infective complications, following surgical vascular grafts or the implantation of prosthetic devices (De Lalla, 1999). Treatment of postoperative infections is further complicated by the emergence of antibiotic-resistant pathogens which has contributed significantly to the morbidity and mortality of hospitalized patients. Most *Staphylococcus* infections result in acute diseases. *Staphylococcus aureus* is a facultative anaerobic, gram positive bacterium, which causes food poisoning and usually grows on the nasal membrane and skin. It is also found in the gastrointestinal and urinary tracts of warm-blooded animals (Cheesbrough, 2000). It also causes boils, abscesses, wound infection, pneumonia, toxic shock syndrome, and other diseases (Cheesbrough, 2000).

S. aureus rapidly develops resistance to many antimicrobial agents. *Staphylococcus epidermidis* is the most common cause of nosocomial bacteremia and is the principal organism responsible for infections of implanted prosthetic medical devices such as prosthetic heart valves, artificial joints, and cerebrospinal fluid shunts (Rupp and Archer, 1994). Resistant bacteria representing a challenge in the treatments of various well-known infections necessitated the need to find new substances with antimicrobial properties to be used in the combat against these microorganisms (Martins et al., 2001).

Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases (Clardy and Walsh, 2004). Current research on natural molecules and products primarily focuses on plants since they can be sourced more easily and selected on the basis of their ethno-medicinal use (Verpoorte et al., 2005). Antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms (Mitscher et al., 1987; Selim, 2011). There are several reports regarding the antimicrobial activity of plant crude extracts and the bioassay-guided fractionation to yield active principles (Palombo and Semple, 2001; Parekh and Chands, 2007a).

The use of traditional antibiotics as food additives has led to the emergence of antibiotic resistant strains of bacteria worldwide (Neu, 1992). On the other hand, an upsurge in cases of histomonosis and other infectious diseases is reported in countries where the use of medical prophylactics is very restricted or is banned in some animals (McDougald, 2005). These observations indicate the need and importance for intensive research in the field of alternative prophylactics and therapeutics. Phytotherapy has become an active area of research in

this scenario (Cowan, 1999). Antimicrobial effects of medicinal plants and their components is being reported with great frequency, however, their effects against antibiotic resistant strains of bacteria, particularly of poultry origin, are less well investigated (Barnes and Bradley, 2003). Alkaloids of plant origin are of special interest for conferring antimicrobial activity and their use in modern medicine (Schmeller and Wink, 1998). The search for components with antimicrobial activity has gained increasing importance in recent times, due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistant microorganisms (Davis, 1982).

However, there has also been a rising interest in the research for natural products from plants for the discovery of new antimicrobial and antioxidant agents in the last three decades and in recent times (Rios and Recio, 2005). More so, many of these plants have been known to synthesize active secondary metabolites such as phenolic compounds in essential oils with established potent insecticidal (Kambu et al., 1982) and antimicrobial activities, which indeed has formed the basis for their applications in some pharmaceuticals, alternative medicines and natural therapies (Rios and Recio, 2005). Santos et al. (1995) remarked that the World Health Organization has indeed recognized medicinal plants as the best source for obtaining a variety of synthetic drugs. No doubt, some studies have identified and isolated the main active ingredients in the plants responsible for this antimicrobial activity (Oloke et al., 1988). However, the study on medicinal plants will allow for the demonstration of their physiological activity and also catalyze many pharmacological studies that will lead to the development of more potent drugs, with no or minimal toxicity and high sensitivity especially towards the emerging microbial agents (Fabricant and Farnsworth, 2001).

Several studies conducted in the past three decades had focused on the antimicrobial properties of herbs, spices and their derivatives such as essential oils, extracts and decoctions (Alma et al., 2003). This research project will deal with studying the effect of essential oils of *Origanum majorana*, *Peganum harmala* and *Salvia officinalis* wild plants used in folk medicine in Egypt to control food pathogenic microorganisms. Also, investigating the changes rate of weight gain and complete blood picture were performed to animals to be sure that administration of large amounts of plant oils is safe or not.

MATERIALS AND METHODS

Plant material and isolation of essential oil

The aerial parts of wild *O. majorana*, *P. harmala* and *S. officinalis* were collected from Ismailia villages, Egypt at full flowering stage. The voucher specimen has been deposited in the Botany Department,

Faculty of Science, Suez Canal University, Egypt. Collected plant materials were dried in shade and ground in a grinder. The dried plant samples (500 g) were subjected to hydrodistillation (plant material in boiling water) using a cleverger-type apparatus for 4 h. Hydrodistillation of plants yielded 2.3% (v/w) of essential oil. The yields were based on dry material of plant sample and stored until analyzed.

Antimicrobial susceptibility testing

Antimicrobial activity tests were performed using broth microdilution methods described by National Committee for Clinical Laboratory Standards (NCCLS) (2008). The medium used was Muller-Hinton broth. The essential oils were prepared in dimethyl sulphoxide (DMSO) and the correct volume was put in the first microplate well with Muller-Hinton broth medium for the concentration of each natural compound to be 250 µg/ml in that well. The cell suspension was prepared in 0.85% saline, with an optical density equivalent to 0.5 McFarland standard and diluted 1:100 in Muller-Hinton broth to obtain a final concentration of 1×10^4 to 5×10^4 colony-forming units per milliliter (CFU/ml). This suspension was inoculated in each well of a microdilution plate previously prepared with the essential oils to give concentrations from 250 µg/ml down to 0.4 µg/ml. The plates were incubated with agitation at 37°C for 24 h for bacterial strains. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which the optical density (OD) was reduced to 90% of the OD in the growth control well as measured by spectrophotometer. For the essential oils, the MIC was defined as the lowest concentration able to inhibit any visible microbial growth. Results were analyzed visually and spectrophotometrically. Extracts displaying an MIC less than 75.0 µg/ml were considered to have strong antimicrobial activity, from 75.0 to 150.0 µg/ml, the antimicrobial activity was moderate, from 150.0 to 250.0 µg/ml, the antimicrobial activity was weak, and over 250.0 µg/ml, the extract was considered inactive.

Gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry analysis (GC-MS) of oils were done at Central Lab in Egyptian Petroleum Research Institute (EPRI), Cairo, Egypt. Analysis of the oils was performed using a Parken Almer (Clarus 500) GC, equipped with a HP 5972 mass selective detector and a HP-5 MS capillary column (30 m × 0.25 mm id, film thickness 0.25 µm). For GC/MS detection, an electron ionization system, with ionization energy of 70 eV, a scan time of 1.5 s and mass range 40 to 300 amu, was used. Helium was the carrier gas at a flow rate of 1.2 ml/min. Injector and transfer line temperatures were set at 250 and 280°C, respectively. Oven program temperature was the same with GC analysis. Diluted samples (1/100 in hexane, v/v) of 1.0 µl were injected manually and in the splitless mode. The identification of the compounds was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library) or with authentic compounds and confirmed by comparison of their retention indices either with those of authentic compounds or with data published in the literature as described by Adams (2001). Further confirmation was done from Kovats Retention Index data generated from a series of n-alkanes retention indices (relative to C9 to C28 on the BP-1) (Hayouni et al., 2008).

Toxic effects of oils

In this experiment, the harmful effects were investigated. Changes in rate of weight gain and complete blood picture were performed

on animals to be sure that administration of large amounts of plants oils is safe. Fifty male hamsters (90 to 250 g) were obtained. After they had been acclimatized for one week, they were maintained throughout the study in a specific pathogen-free environment with a temperature of $24 \pm 2^\circ\text{C}$, a humidity of 60 ± 15 and a 12 h light-dark cycle. Hamsters were housed in clean aluminum cages, each of which held 5 hamsters, and they were provided with the pellet diet CE-2 and water *ad libitum*. In this experiment, ten groups of five rats were used each: group 1 (control treated with saline), groups 2, 3, 4 (treated with 80,160 and 320 mg/kg of *S. officinalis* oil, respectively), groups 5, 6, 7 (treated with 80,160 and 320 mg/kg of *O. majorana* oil, respectively) and group 8, 9, 10 (treated with 80,160 and 320 mg/kg of *P. harmala* oil, respectively). Commercial oils were suspended in saline solution (SS) and were administered orally by intragastric route. The experiment continued for 14 days. Body weights were recorded every 3 days (Nagayama et al., 2002).

Statistical analysis

The variations between experiments were estimated by standard deviations, and statistical significance of changes was estimated by student's t-test. Only the probability $P \leq 5\%$ was regarded as indicative of statistical significance.

RESULTS

The patterns of antibacterial effect of oils from *O. majorana* were detected after treatment of the studied bacterial species (Table 1). Moderate effect was observed at all concentration on *Bacillus cereus*. Weak effect was detected at all concentrations of this oil on *Salmonella indica* and *S. aureus*. No effect (-) was recorded at concentrations of 250 µg/ml of this oil after treatment of *Escherichia coli*. The chemical composition of the commercial oil of *O. majorana* was determined by GC/MS method. Seventeen compounds (Table 2 and Figure 1a) were identified, and the following compounds are representing the major constituents: (1) 1,30-dibromotriacontane (41.07%), (2) 11-tricosene (15.35%), (3) 1,38-dibromooctatriacontane (9.78%), (4) 1-pentacontanol (9.05%), (5) O-2-methylpropylhydroxylamine (7.02%), (6) 2-piperidinone,N-(4-bromo-N-butyl) (6.39%), (7) 2-methyl-tricosane (3.07%) and (8) 9-cyclohexyl-eicosane (2.17%), respectively.

The effect of *P. harmala* oil on the growth of *B. cereus*, *S. indica*, *S. aureus* and *E. coli* are shown in Table 1. The obtained results revealed that concentrations (75 µg/ml) of *P. harmala* oil had the highest antibacterial activity against *B. cereus* and *S. aureus*. The concentration of 250 µg/ml was not effective against *S. indica*. The chemical composition of the commercial oil of *P. harmala* was determined by GC/MS method. Forty one compounds (Table 3 and Figure 1b) were identified and the following compounds are representing the major constituents: (1) 1-hexyl-2-nitrocyclohexane (9.07%), (2) Z-2-octadecan-1-ol (8.13%), (3) 3,5,24-trimethyltetracontane (7.84%), (4) 2-octadecyl-1,3-propane-diol (6.18%), (5) E-2-tetradecen-1-ol (5.89%), (6) 11,14-ecosadienoic acid

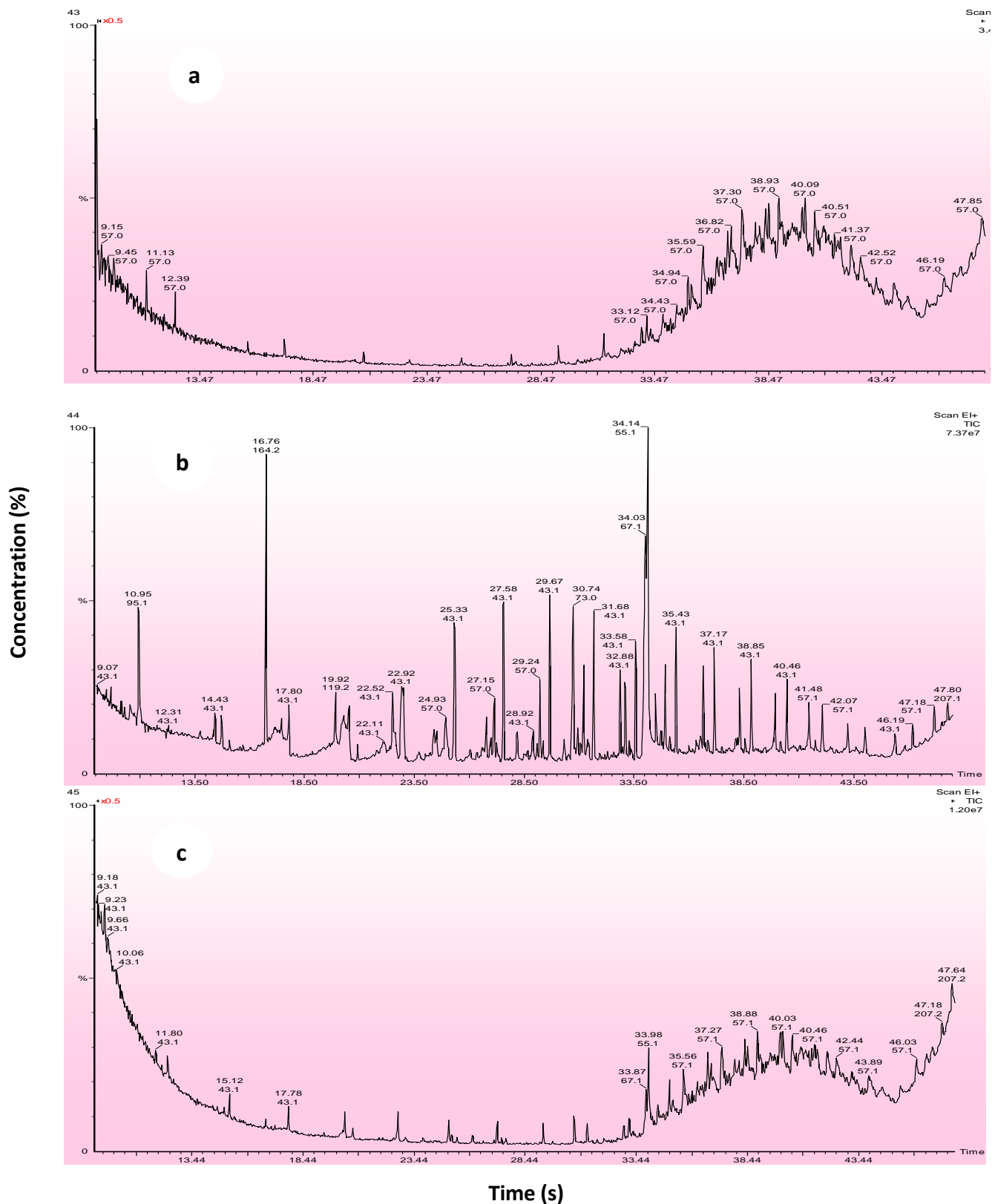


Figure 1. Gas chromatographic profile of the major constituents of (a) *Origanum majorana*, (b) *Peganum harmala* and (c) *Salvia officinalis* oils.

Table 1. Antimicrobial susceptibility of *Origanum majorana*, *Peganum harmala* and *Salvia officinallis* oils against studied bacterial species.

Plant species	Bacterial species			
	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>	<i>Salmonella indica</i>	<i>Escherichia coli</i>
<i>Origanum majorana</i>	M	W	W	N
<i>Peganum harmala</i>	S	S	N	M
<i>Salvia officinallis</i>	W	W	W	N

Where MIC less than 75.0 µg/ml was considered to have strong antimicrobial activity (S), from 75.0 to 150.0 µg/ml, the antimicrobial activity was moderate (M), from 150.0 to 250.0 µg/ml, the antimicrobial activity was weak (W), and over 250.0 µg/ml, the extract was considered inactive (N).

Table 2. Chemical composition (%^a) of the commercial oil of *Origanum marjorana*.

Compound	MW	Formula	RT	wt %
1,30-Dibromotriacontane	578	C ₃₀ H ₆₀ Br ₂	41.85827	41.07
11-Tricosene	322	C ₂₃ H ₄₆	38.86413	15.346
1,38-Dibromooctatriacontane	690	C ₃₈ H ₇₆ Br ₂	41.666	9.777
1-Pentacontanol	718	C ₅₀ H ₁₀₂ O	36.2113	9.046
O-2-Methylpropyl-hydroxylamine	89	C ₄ H ₁₁ ON	11.391	7.023
2-Piperidinone, N-[4-bromo-N-butyl]	233	C ₉ H ₁₆ ONBr	33.71163	6.391
2-Methyl-tricosane,	338	C ₂₄ H ₅₀	29.721	3.071
9-Cyclohexyl-eicosane,	364	C ₂₆ H ₅₂	37.01467	2.173
1,54-Dibromo-tetrapentacontane,	914	C ₅₄ H ₁₀₈ Br ₂	38.37167	1.493
2,4,6-Tritert-butyl-4-methyl-2,5-cyclohexadien-1-one	276	C ₁₉ H ₃₂ O	21.659	1.441
1-Hexyl-2-nitrocyclohexane	213	C ₁₂ H ₂₃ O ₂ N	33.323	0.719
2-Azido-2,3,3-trimethylbutane	141	C ₇ H ₁₅ N ₃	15.553	0.699
6-Ethyl-2-methyl-decane	184	C ₁₃ H ₂₈	26.7075	0.598
N-(1-phenyl-2-propanyl)-1-decanamine	275	C ₁₉ H ₃₃ N	27.177	0.597
DL-3,4-dimethyl-3,4-hexanediol	146	C ₈ H ₁₈ O ₂	25.7035	0.308
Taurolidine	284	C ₇ H ₁₆ O ₄ N ₄ S ₂	10.999	0.126
2-Aminononadecane	283	C ₁₉ H ₄₁ N	28.087	0.121
Identified components (%)				100

%^a peak area of oil components. MW = Molecular weight, RT = Retention time.

methyl ester (5.79%), (7) eugenol (5.22%), (8) 2,6,10,15-tetramethyl-heptadecane, (4.26%), (9) 11-tricosene (4.10%), (10) 2-piperidinone,N-(4-bromo-N-butyl) (3.49%), (11) L-(+)-ascorbic acid 2,6-dihexadecanoate (3.47%), (12) 14-heptadecenal (2.87%), (13) E-9-tetra decenoic acid (2.77%), (14) 1,1-dodecanediol, diacetate (2.64%) and (15) 2-methyl-7-octadecyne (2.45%), respectively.

Two patterns were observed after treatment of *S. officinallis* oil on the studied bacterial species. No effect (N) and weak effect (W). The first one, no effect, was recovered in *E. coli* after treatment with concentration (250 µg/ml) of *S. officinallis* oil (Table 1). The weak effect was also recorded in *B. cereus*, *S. aureus* and *S. indica* at all concentrations of *S. officinallis* oil. The chemical composition of the commercial oil of *S. officinallis* was

determined by GC/MS method. Seventeen compounds (Table 4 and Figure 1c) were identified and the following compounds are representing the major constituents: (1) Docosanoic acid, docosyl ester (16.63%), (2) 2-piperidinone,N-(4-bromo-N-butyl) (15.02%), (3) DL-3,4-dimethyl-3,4-hexanediol (12.82%), (4) 1,30-dibromotriacontane (10.91%), (5) 11-tricosene (9.03%), (6) 1-pentacontanol (6.50%), (7) 1,38-dibromo-octatriacontane (5.86%), (8) heptane,4-azido (4.15%), (9) 3-ethyl-5-(2-ethylbutyl)-octadecane (4.11%), (10) 3-bromo-decane (3.07%) and (11) 1-hexyl-2-nitrocyclohexane (2.43%), respectively.

Table (5) shows the effect of 80, 160 and 320 mg/kg concentration from different studied oils on hamsters. The increase in body weight was observed in days 3, 6 and 9 when animals were treated with 160 mg/kg *O. majorana*

Table 3. Chemical composition (%^a) of commercial oil of *Peganum harmala*.

Compound	MW	Formula	RT	wt %
1-Hexyl-2-nitrocyclohexane	213	C ₁₂ H ₂₃ O ₂ N	34.087	9.072
Z-2-Octadecen-1-ol	268	C ₁₈ H ₃₆ O	30.88133	8.125
3,5,24-Trimethyltetracontane	604	C ₄₃ H ₈₈	37.48822	7.84
2-Octadecyl-1,3-propane-diol	328	C ₂₁ H ₄₄ O ₂	28.502	6.181
E-2-Tetradecen-1-ol	212	C ₁₄ H ₂₈ O	28.2885	5.893
11,14-Eicosadienoic acid methyl ester	322	C ₂₁ H ₃₈ O ₂	34.033	5.787
Eugenol	164	C ₁₀ H ₁₂ O ₂	16.758	5.216
2,6,10,15-Tetramethyl-heptadecane,	296	C ₂₁ H ₄₄	29.81033	4.261
11-Tricosene	322	C ₂₃ H ₄₆	25.97133	4.097
2-Piperidinone, n-[4-bromo-n-butyl]	233	C ₉ H ₁₆ ONBr	32.403	3.486
l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	C ₃₈ H ₆₈ O ₈	30.712	3.465
14-Heptadecenal	252	C ₁₇ H ₃₂ O	42.193	2.875
E-9-Tetradecenoic acid	226	C ₁₄ H ₂₆ O ₂	34.167	2.765
1,1-Dodecanediol, diacetate	286	C ₁₆ H ₃₀ O ₄	17.186	2.64
2-Methyl-7-octadecyne	264	C ₁₉ H ₃₆	10.973	2.448
2,6,10,14-Tetramethyl-heptadecane,	296	C ₂₁ H ₄₄	24.927	1.954
E-3-Pentadecen-2-ol	226	C ₁₅ H ₃₀ O	37.193	1.679
16-Heptadecenal	252	C ₁₇ H ₃₂ O	38.854	1.576
2-Dodecyl-oxirane,	212	C ₁₄ H ₂₈ O	17.776	1.446
E-15-Heptadecenal	252	C ₁₇ H ₃₂ O	32.881	1.323
DI-3,4-Dimethyl-3,4-hexanediol	146	C ₈ H ₁₈ O ₂	16.3454	1.264
9-Octyl eicosane	394	C ₂₈ H ₅₈	44.3045	1.228
1-(1,5-Dimethyl-4-hexenyl)-4-methylbenzene	202	C ₁₅ H ₂₂	19.918	0.967
6,10,14-Trimethyl-2-pentadecanone	268	C ₁₈ H ₃₆ O	28.194	0.82
Docosanoic acid, docosyl ester	648	C ₄₄ H ₈₈ O ₂	36.122	0.738
Taurolidine	284	C ₇ H ₁₆ O ₄ N ₄ S ₂	14.401	0.704
Heptafluorobutyric acid,n-tridecyl ester	396	C ₁₇ H ₂₇ O ₂ F ₇	34.515	0.689
9-Ethyl-9-heptyl octadecane	380	C ₂₇ H ₅₆	47.184	0.628
1,1-Dodecanediol, diacetate	286	C ₁₆ H ₃₀ O ₄	14.722	0.616
1-Pentacontanol	718	C ₅₀ H ₁₀₂ O	34.89	0.519
4-Methyl cyclopentadecanone	238	C ₁₈ H ₃₆ O	30.337	0.458
DI-3,4-dimethyl-3,4-hexanediol	146	C ₈ H ₁₈ O ₂	23.695	0.408
Heptafluorobutyric acid, n-octadecyl ester	466	C ₂₂ H ₃₇ O ₂ F ₇	30.953	0.364
2,6,10,14-Tetramethyl heptadecane	296	C ₄₃ H ₈₈	22.65	0.329
Hexadecanoic acid, (3-bromoprop-2-ynyl) ester	372	C ₁₉ H ₃₃ O ₂ Br	33.337	0.321
Oxirane, 2-decyl-3-(5-methylhexyl)-, cis	282	C ₁₉ H ₃₈ O	36.363	0.27
2-Nonadecanone	282	C ₁₉ H ₃₈ O	27.284	0.217
Heptadecyl ester heptadecanoic acid	508	C ₃₄ H ₆₈ O ₂	38.131	0.169
DI-3,4-dimethyl-3,4-hexanediol	146	C ₈ H ₁₈ O ₂	17.7555	0.113
Eicosanoic acid	312	C ₂₁ H ₄₄ O ₂	26.587	0.096
Heptane, 4-azido	141	C ₇ H ₁₅ N ₃	10.598	0.504
Identified components (%)				99.67

%^a peak area of oil components. MW = Molecular weight, RT = Retention time.

oil. A gradual increase in body weight was recorded after treatment with 80 mg/kg *P. harmala* oil at 3, 6, 9, 12 and 14 days. The weight increase of hamster was recorded at days 3 and 6 after treatment with 160 mg/kg of *P.*

harmala. Reduction in body weight was observed in this group which was treated with dose 160 mg/kg in only day 9. At days 12 and 14, the group returns to increase in its body weight more than the control group. The obtained

Table 4. Chemical composition (%^a) of commercial oil of *Salvia officinalis*.

Compound	MW	Formula	RT	wt %
Docosanoic acid, docosyl ester	648	C ₄₄ H ₈₈ O ₂	43.45	16.625
2-Piperidinone, n-[4-bromo-n-butyl]-	233	C ₉ H ₁₆ ONBr	34.6328	15.022
DI-3,4-Dimethyl-3,4-hexanediol	146	C ₈ H ₁₈ O ₂	24.75505	12.821
1,30-Dibromo- triacontane,	578	C ₃₀ H ₆₀ Br ₂	39.9655	10.906
11-Tricosene	322	C ₂₃ H ₄₆	38.24571	9.027
1-Pentacontanol	718	C ₅₀ H ₁₀₂ O	37.40014	6.497
1,38-Dibromo-octatriacontane,	690	C ₃₈ H ₇₆ Br ₂	41.13075	5.856
Heptane, 4-azido-	141	C ₇ H ₁₅ N ₃	12.20945	4.153
3-Ethyl-5-(2-ethylbutyl)-octadecane,	366	C ₂₆ H ₅₄	44.7088	4.11
3-Bromo-decane	220	C ₁₀ H ₂₁ Br	26.065	3.07
1-Hexyl-2-nitrocyclohexane	213	C ₁₂ H ₂₃ O ₂ N	34.12	2.434
2-Methyl-tricosane	338	C ₂₄ H ₅₀	31.06	1.929
o-(2-Methylpropyl)-hydroxylamine	89	C ₄ H ₁₁ ON	9.928	1.802
1-(2-Decyldodecyl)-2,4-dimethyl- cyclopentane	406	C ₂₉ H ₅₈	40.555	1.147
2,3,4,5,6,7-hexahydro-3,6-dihexyl-10,11-diphenyl- bis[1,3]oxazino[6,5-f:5',6'-h]quinoxaline	564	C ₃₆ H ₄₄ O ₂ N ₄	15.3605	1.057
Di-n-undecylamine	325	C ₂₂ H ₄₇ N	26.078	0.665
3,5,24-Trimethyltetracontane	604	C ₄₃ H ₈₈	36.852	0.538
Identified components (%)				99.96

%^a peak area of components. MW = Molecular weight, RT = Retention time.

Table 5. Body weight (g) of hamsters treated with potent oils (mg/kg) for 14 days and percentage of weight gain.

Day	Weight (g)	Control	<i>Origanum majorana</i>			<i>Peganum harmala</i>			<i>Salvia officinalis</i>		
		0	80	160	320	80	160	320	80	160	320
0	Weight	247.75	221.25	130.31	177.1	148	157.72	210.48	90.52	137	165.66
3	Weight	241.16	185.51	131.2	156.88	163.77	159.9	199.37	92.82	138.12	160.19
	Weight gain (%)	-2.66	-16.15	0.68	-11.42	10.66	1.38	-5.28	2.54	0.82	-3.3
6	Weight	264.54	196.4	132.5	150.12	170.92	160.88	190.2	95.9	140.29	157.84
	Weight gain (%)	6.78	-11.23	1.68	-15.23	15.49	2	-9.64	5.94	2.4	-4.72
9	Weight	253.9	199.8	133.43	143.44	180.96	151.09	183.48	99.93	141.11	152.93
	Weight gain (%)	2.48	-9.69	2.39	-19	22.27	-4.2	-12.83	10.39	3	-7.68
12	Weight	250.8	202.08	127.52	141.83	174.3	158.5	200.36	100.3	130.55	135.57
	Weight gain (%)	1.23	-8.66	-2.14	-19.91	17.77	0.49	-4.81	10.8	-4.7	-18.16
14	Weight	240.42	215.68	129.47	140.86	158.42	167.8	181.13	102.25	144.3	163.75
	Weight gain (%)	-2.96	-2.52	-0.64	-20.46	7.4	6.39	-13.94	12.96	5.33	-1.15

data showed that the administration by high dose of *S. officinalis* (320 mg/kg) reduced body weight of hamsters at days 3, 6, 9, 12 and 14. The decreasing effect of 160 mg/kg of and *S. officinalis* on hamsters was observed

only at day 12, while the weight gain was observed in days 3, 6, 9 and 14 (Table 5). In general, the hamsters animal demonstrated both slight increase or decrease in animal body weight after treatment with 80, 160 and 320

Table 6. Hematological parameters of blood of hamsters treated with oils (mg/kg) after 14 day.

Group	Normal value	control	<i>Origanum majorana</i>			<i>Peganum harmala</i>			<i>Salvia officinalis</i>		
		0	80	160	320	80	160	320	80	160	320
RBCs	9-15×10 ⁸ g/l	4.9	4.58	5.53	4.53	5.52	5.47	5.41	6.8	6.84	6.46
HGB	9-15 g/dl	11.6	11.4	11.9	11.2	12.2	11.4	11.6	12.5	14.7	12.8
HCT%	27-45	42.8	37	42.6	36.9	45.8	38.1	39.4	43.6	48.3	43.9
MCHC	31-34 g/dl	27.1	30.9	28	30.2	26.5	29.9	29.5	28.7	30.9	29.2
WBCs	4-12×10 ⁸ g/l	8.38	4.46	4.1	5.3	20.2	4.02	7.64	6.61	12.8	3.06
lymphocyte	2-9×10 ⁸ g/l	3.66	1.35	0.99	1.03	2.44	1.22	3.04	2.07	2.46	0.73
Lymphocyte%	40-75	43.7	30.2	24.3	19.3	12.1	30.2	39.8	31.3	19.2	23.8
Monocyte	ND	0.06	0.04	0.04	0.05	1.2	0.03	0.17	0.23	0.11	0.03
Monocyte%	0-5%	0.8	0.8	0.9	0.9	5.9	0.8	2.2	3.4	0.9	0.9
Granulocyte	0.70-6.00	4.66	3.08	3.07	4.23	16.6	2.77	4.42	4.32	10.2	2.31
Granulocyte%	10.0-50.0	55.6	68.9	74.9	79.8	82	68.9	57.9	65.3	80	75.3

ND; not detected.

mg/kg, which was statistically insignificant with oils of *O. majorana*, *P. harmala* and *S. officinalis* within time of treatment.

The effect of different doses of *O. majorana*, *P. harmala* and *S. officinalis* oils on total counts of red blood cells (RBCs), white blood cells (WBCs) and other hematological parameters after 14 days of treatment on hamsters were recorded in Table 6. It is of interest to note that all treatments of *O. majorana*, *P. harmala* and *S. officinalis* oils had increasing effect on RBCs, haemoglobins (HGB) and hematocrit (HCT%). The maximum values of RBCs (6.84 g/l), HCT% (48.3%) and HGB (14.7%) were recorded after the treatment with 160 mg/kg of *S. officinalis*. All values of mean cell hemoglobin concentration (MCHC) were increased in all treatment by 80, 160, 320 mg/kg of *O. majorana*, *P. harmala* and *S. officinalis* oils. The concentration of 160 mg/kg of *S. officinalis* had a good effect on MCHC value (30.9).

In general, all doses of *O. majorana* and *S. officinalis* oils reduced the total counts of WBCs of treated hamsters compared to untreated animal except 160 mg/kg of *S. officinalis*. There were remarkable reduction in lymphocytes and lymphocytes percentage as a result of treatment with all doses of oils at days 3, 6, 9, 12 and 14. No change in monocyte percentage was observed in case of treatment with 80 mg/kg of *O. majorana* and 160 mg/kg of *P. harmala*. Slight increase (0.1%) was observed after the treatment with 160 and 320 mg/kg from both *O. majorana* and *S. officinalis*. The highest increase in monocyte values were 5.9, 3.4 and 2.2% after the treatment by 80 mg/kg *P. harmala*, 80 mg/kg *S. officinalis* and 320 mg/kg *P. harmala*, respectively. All values of granulocyte percentage were increased in all treatment. The assumption of weight gain results is supported by hematological analysis (Table 6).

DISCUSSION

Many herbs, essential oils and species have demonstrated some inhibitory effect against spoilage microorganisms in variety of foods (Ellin, 2007). Also, herbs are used as substances enhancing the taste and varieties of regular foods. Some herbs are used as meat additives which have been reported to have bactericidal or bacteriostatic additives (Dyankova et al., 2009). The inhibitory effects of herbs are mostly because of their content of volatile oils (Vazgecer et al., 2004). Many medicinal plants have been known to synthesize active secondary metabolites such as phenolic compounds found in essential oils, with established potent insecticides and antimicrobial activities (Kambu et al., 1982). Many research groups screened various plants extracts as secondary metabolites to detect their biological activities. Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus they can be used in the treatment of infectious disease caused by resistant microbes. The essential oils of plants are used as antioxidant, laxatives erosive and in treatment of skin, sleep, cold, cough, urinary system, and nervous system disorders (Abo-Ghaila et al., 2004).

This investigation was directed to study the effect of three essential plant oils on the growth of most potent proteolytic bacterial species *B. cereus*, *E. coli*, *S. indica* and *S. aureus*. All oils were tested for their antimicrobial activity using microdilution method. The results revealed that extracts of all plants oils had variations in antibacterial activity against *B. cereus*, *E. coli*, *S. indica* and *S. aureus*. It also revealed that *P. harmala* essential oils have a highest antibacterial activity against the studied bacterial species. The plant extracts and the essential oils of plants exhibited various reduction in the growth

according to its chemical composition. This assumption is in accordance with Rota et al. (2004) who reported that the bacterial effect of thymus oils are supposed to be associated with high levels of carvacrol and linalool.

Kalemba and Kunicka (2003) reported that, because of the great number of cell constituents, volatile oils seem to have no specific cellular targets. As typical lipophilic, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layer of polysaccharides, fatty acids, phospholipids and permeabilized them. The mode of action of antimicrobial agents also depends on the type of microorganisms and their cell wall structure and outer membrane arrangement. Cytotoxicity appears to include such membrane damage. In bacteria, the permeabilization of the membranes is associated with loss of ions, reduction of membrane potential, collapse of the proton pump and depletion of the adenosine triphosphate (ATP) pool (Turina et al., 2006). Moreover the volatile oils can act as pro-oxidant affecting inner cell membranes and organelles such as mitochondria in eukaryotic organisms. Mitochondria like structure and membranes structure in prokaryotic are affected by volatile oil like mitochondria in eukaryotic organisms. Depending on type and concentration, essential oils exhibit cytotoxic effect on living cell but are usually non genotoxic (Bakkali et al., 2008). The antibacterial activity of *O. saccatum* oil was established by using agar diffusion method. Ozcan and Chalchat (2009) showed that *O. saccatum* essential oil exhibits antibacterial activity against *S. aureus* and *E. coli*.

GC/MS analysis had revealed various compounds in the commercial oils of the three plant species studied (17 *O. majorana*, 41 *P. harmala* and 17 *S. officinalis*). GC/MS analysis of oil components revealed many differences between plant species (*Origanum vulgare* and *O. majorana*) while those in *O. majorana* were terpinene-4-ol, α and γ -terpinenes, terpinoline, α and β pinenes, saninene, 1,8 cineol, camphene, β -caryophyllene, α -terpineol and camphene (Novak et al., 2003). The differentiation in the composition between the *O. majorana* is possible; this may be due to biochemical transformation during distillation. It may play a considerable role (Fischer et al., 1987). It might reflect the differences between terpenoid component of the intact plant and composition of distilled *O. majorana* oil (Novak et al., 2003). *Origanum* species showed a strong antibacterial activity against both gram positive and gram negative bacteria (Neslihan et al., 2009). The result showed that antibacterial activity increased depending on the concentration used (Ozcan and Chalchat, 2009). This result is in agreement with obtained result.

P. harmala showed a high antibacterial activity, this result is in agreement with Arshad et al. (2008) who found that *P. harmala* inhibits the growth of all bacteria. Several reports in the literature indicate a great variety of pharmacological activities of *P. harmala* such as anti-

microbial, antitumor, antinociceptive and monoamine oxidase (MAO)-inhibiting activities (Shahverdi et al., 2008). The most important component from *P. harmala* seeds are harmine, harmaline, vasicinone and deoxyvinsone (Astulla et al., 2002). These compounds exhibited various bioactivities such as antibacterial activity (Gaviraj et al., 1998) and enzyme inhibition (Sobhani et al., 2002). All the fractions of *P. harmala* showed a good activity against *S. aureus*. The methanolic fraction was the most active against all organisms tested followed by the chloroform fraction (Prashanth and John, 1999). These results are in agreement with the obtained results. Moreover, Shahverdi et al. (2008) reported that the smoke from burning *P. harmala* seeds reduce the viability of tested microorganisms.

S. officinalis is commonly used in traditional medicine to treat various microbial infections. The extracts of *S. officinalis* showed activity against all gram positive and gram-negative bacteria (Kamatou et al., 2007a). The active compounds in *Salvia* spp. extracts are carnosol, 7-o-methylepirosmanol, oleanolic acid and its isomer ursolic acid. These active compound displayed moderate to good activity against all the gram positive and gram negative bacterial strains. The essential oils of *Salvia* species however displayed moderate activity against gram positive bacteria. Gono-Byola (2003) demonstrated that methanolic extracts and essential oils of three species of *Salvia* collected at various localities in South Africa inhibited the growth of gram positive bacteria. These results and observation of Kamatou et al. (2007a) are in agreement with the obtained results.

Our results revealed that the change (increasing or decreasing) in body weights of hamster exhibited insignificant variations compared to the control group. Besides, no toxic symptoms or death were observed and they survived being active and healthy, up to 14 days. These results are matching with Abdallah et al. (2009) who reported these observations up to 15 days. These findings could be a good indicator for the non toxicity and safety of *O. majorana*, *P. harmala* and *S. officinalis* at doses 80, 160, 320 mg/kg body weight per day.

Ramirez et al. (2007) did not show any significant variation in both treated and untreated of both sexes of rats after the treatment with *Salvia scutellarioides*, our result confirmed this finding. This action of *S. scutellarioides* was similar with our results. The hamsters demonstrated slight increase and decrease in body weights as shown in Tables 3 and 4. These results are in accordance with Tripathi et al. (2006) who found that the experimental animal demonstrated slight increase in body weight which was statistically insignificant, and he claimed that this effect of oil may be due to its non nutritional value. The body weight of the animals treated with hydroalcoholic extract once a day during 15 days did not show any significant change when compared with the control group, although this had a tendency to decrease

body weight at high concentration of 2000 and 4000 mg/kg (Costa et al., 2011). Costa et al. (2011) also found that the amount of weight loss in animals may be directly related to food ingestion.

Pieme et al. (2006) found that the hydroethanolic extract of *Senna alata* leads to progressive weight gain in rats during 26 days. The administration of aqueous ethanol extract of *S. alata* may indicate an improvement in the nutritional state of animal. The growth response effect could be as a result of increased food and water intake. This explanation is in accordance with our results. The assumption of weight gain results is supported by hematological analysis. The daily oral treatment of *O. majorana*, *P. harmala* and *S. officinalis* oils for 14 days showed no significant variation for RBCs and WBCs. In general, the results showed that the value for the RBCs and WBCs were slightly increased or decreased compared with the untreated animals. These variations were not dose dependent. These results are in line with Pieme et al. (2006) who found that hydroalcoholic extract of *S. alata* has a slight effect on some hematological parameters. The insignificant increase or decrease in hematological parameters was recorded by Abdallah et al. (2009). This observation was similar to our results. The increase in HGB content may be due to the effect of oil, Tripathi et al. (2006) reported this finding after treatment of hamsters with oils. Our results showed that hematological estimation for 14 days revealed an increase of RBCs, HGB and granulocyte%. These results are in accordance with Tripathi et al. (2006) who found that the treatment of oitiver oil (gramineae) revealed the increase in HGB also.

Conclusions

In summary, our study demonstrated that *O. majorana*, *P. harmala* and *S. officinalis* oils seem to be destitute of toxic effect which could compromise the medicinal use of these plants in folk medicine. More detailed study in the future is necessary to clarify exactly the safety of plants extracts and plants oils for human.

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