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Full Length Research Paper

Spermicidal and antigonococcal effects of tannins from pomegranate rind

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The purpose of this work was to evaluate the spermicidal, antigonococcal and antifertility efficiency of tannins in pomegranate rind (TPR). Spermicidal activity was assessed by a modified version of the Sander-Cramer test and World Health Organization (WHO) standard method. A chromatographic method was applied to screen the spermicidal and antigonococcal ingredients of TPR, and the antifertility effect was investigated using rabbits. Spermicidal effect of TPR was identified both *in vitro* and in rabbit. The minimum effective spermicidal concentration of TPR *in vitro* was 1.25 mg/ml. In antigonococcal process, TPR shows moderate performance. However, TPR terminated the origination of pregnancy significantly in rabbit.

Key words: Spermicide, gonorrhoeae, tannins; pomegranate rind.

INTRODUCTION

With constant increases of global population, it is imperative that birth-control policy should be a major concerned. Even though a variety of contraceptive agents are on sale, their mechanisms of prevenception mainly include antifertilization, anti-implantation and inhibition of ovulation. Some side effects, such as colporrhagia, body weight gain and depressive disorders, which threaten female physiological and mental health, are observed in usage frequently. Owing to the risk of causing irreversible azoospermatism and other adverse reactions, it is difficult to generalize and apply for male contraceptives, such as gossypol. Therefore, it is a stimulating challenge for current pharmaceutical scientists to find and utilize natural products or derivatives as contraceptives with simplicity, low toxicity and hyper efficiency. *Neisseria gonorrhoeae* infection is a common bacterial of sexually transmitted disease. Though various types of preventive antibiotics have been developed over the last several years, *N. gonorrhoeae* strains have developed a high level of resistance to several antibiotics because of misuse and irregularity in the treatment (Mbwana et al., 1999). The treatment has become more complicated particularly due to multiple drug resistance. Pomegranate rind is a shrub, which was distributed originally in Iran and Afghanistan, and introduced into China in the second century BC.

It is cultivated throughout the world, mainly grown in the Near East, India, Spain (southeastern), Israel and the United States (California). In traditional Chinese medicines, Pomegranate Rind has been used to treat dysentery, microbial infections, diarrhea and haemorrhage (Negi et al., 2003). The main content of Pomegranate Rind is tannin, as a kind of polyphenols, which takes 10.4-21.3% in weight. These natural tannins have a variety of biochemical and pharmacological properties (Joseph et al., 2012; Dell'agli et al., 2010). It's probable that some certain actions may occur between tannins and sperm, which based on the common tannin-protein reaction. Recently, the relevant reports showed that tannins in Pomegranate rinds exhibited inhibition on sperm motility (Parkhuyst and Stolzenbberg, 1974). The extract of Pomegranate Rind was recognized a useful source of highly active antigonorrhoeal metabolites (Gibbons, 2005). Pomegranate Rind contain plenty of tannins, thus, this assay was conducted to find some scientific evidence of the tannins for antigonorrhoeal activity. In this paper, the inhibition of

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Pomegranate Rind on sperm motility was determined and the active parts of the tannin in Pomegranate Rind inhibiting the sperm motility were screened in order to find potent inhibition and low side effects active parts, and the antifertility of Pomegranate rinds was tested in rabbits by intravaginal administration. The present study was also aimed at evaluating the antigonorrhoeal activities of six fractions of extracts from Pomegranate Rind (Silva et al., 1997).

MATERIALS AND METHODS

Plant material

Pomegranate Rind were obtained from Xinjiang Province (China) in November 2007 and identified by Professor Hong Zhang (College of Pharmacy, Wuhan University, Wuhan, China).

Apparatus and reagents

Sepabeads HP-20 balsam; Sodium Chloride; Nonoxynol-9 and tannic acid from Chinese Gall were purchased from Sigma Chemical Company St. Louis, USA. Acetone and methanol were of analytical grade. Gas chromatography (GC) agar powder (Oxiod, British), haemoglobin (Oxiod, British), Vitox supplement (Oxiod, British), beef extract, enzymatic digestion casein and amylum solubile.

Animals and gonorrhoeae

Mature New Zealand rabbits (7 to 10 months old) weighing 2.5 to 3.0 kg were purchased from Wuhan Institute of Biologic Products (Wuhan, China). Female and male rabbits were kept under standard environmental conditions, respectively, and fed with diet and water ad libitum. Three WHO control strains B (29501), W1 (29400), W2 (29403) were used in the present study.

Preparation of TPR

Pomegranate Rind was purchased from Wuhan Medicinal Material Corporation. Dried pomegranate rinds were ground in a cutting mill fitted with a 40 mesh screen at the bottom of the cutting chamber. A certain amount of comminuted pomegranate rinds was soaked in distilled water for 30 min. The residua were extracted with 8～10 volumes of distilled water for 10 h at room temperature. Then the residua were filtrated out and concentrated by a rotary evaporator at 40℃. The partial concentrated solution was cryodesiccated and colored solid was obtained for phenolic determination. According to the equation:

$$C = \frac{g}{v} \times 100\%$$

Where c is the concentration of TPR, g represents the weight of pomegranate rinds; v refers to the volume of TPR.

Preparation and fractionation of TPR

10.0 g of comminuted pomegranate rinds were extracted with 3 volumes of 70% acetone (v/v) for 6 h at room temperature, then in an ultrasonic bath for 0.5 h. After that, the residua were filtrated out and the operations were repeated 3 times. The combined extracts were concentrated by using a rotary evaporator at 40℃ under reduced pressure. Then the residue was separated by column chromatography (CC) on HP-20 using distilled water, 10% methanol (v/v), 20% methanol, 40% methanol, 60% methanol, 70% acetone as eluents by turns to yield 6 fractions. Fractions were cryodesiccated and phenolics determined by the method as describe, respectively.

Phenolics determination

Phenolics in the TPR and fractions of pomegranate rinds (FPR) were determined by the method of literature (Taga et al., 1984). The concentrated fractions were filtered through a 0.45 μm Millipore filter. To 500 μl filtrate, 500 μl of Folin–Ciocalteau reagent (50%, v/v) and 10 ml of sodium carbonate (2%, w/v) were added and mixed, respectively. After 2 h, the absorbance of the solution was measured at 750 nm. Quantitation was based on the standard curve of gallic acid (0 to 1.0 mg/ml), dissolved in methanol/water (60: 40, v/v; 0.3% HCl). Phenolic content was expressed as milligrams per gram of equivalent of gallic acid.

Human semen collection

The semen samples provided by medical center of GEN, Renming Hospital of Wuhan University were collected by masturbation from five nondrinker and nonsmoker male volunteers (20 to 25 years) with a mean spermatozoal count of $> 20 \pm 0.5 \times 10^6$ spermatozoa/ml and $> 70 \pm 2\%$ normal sperm morphology (Reynolds and Narang, 1984). The semen characteristics, volume, pH, viscosity and sperm morphology were determined according to World Health Organization guidelines. Samples were collected into a warm glass beaker to avoid cold shock to the sperm. The fresh samples were allowed to liquefy and further investigations were carried out at 35℃.

The inhibition of TPR on sperm motility

The experiment of pomegranate rinds on inhibiting the sperm motility *in vitro* was determined by following a modified version of the Sander-Cramer test (Khillare and Shrivastav, 2003). 0.2 ml of liquefied semen was kept in a water bath at 37℃. Different concentrations of TPR in physiological saline (1.0, 2.05 and 4.0%) were prepared by serial dilution and 100 μl of each was mixed with an aliquot of 20 μl of sperm suspension (2.5×10^7 ml^{-1}). Rapidly the mixtures were observed under a phase contrast microscope (×100) for 20 s and analyzed for percentage and grade of sperm motility (Shrabanti et al., 2007). NP-9 was used as reference substance.

The spermicidal activity of TPR

The experiment of inhibiting the sperm motility (Fu et al., 2003) *in vitro* was carried out according to WHO standard method (WHO, 1992). Different concentrations of TPR in sodium chloride (5.0000, 2.5000, 1.2500, 0.6250 and 0.3125 $mg \cdot ml^{-1}$) were prepared by serial dilution. 0.5 ml of each was mixed with an aliquot of 0.1 of ml sperm suspension. Rapidly the mixtures were observed under a phase contrast microscope (×100) at (25 ± 2)℃ for 20 to 180 s and analyzed for percentage and grade of sperm motility. Different concentrations of NP-9 (0.5000, 0.2500, 0.1250 and 0.0625 mg/ml) and sodium chloride were used as control.

Table 1. Agglutination of human semen by pomegranate rinds ($\overline{X}$±SD) (n=20).

Group	Concentration (mg/ml)	Time of agglutination (s)
TPR A	1.0	30±3.16*
TPR B	2.0	10±2.98**
TPR C	4.0	8.0±1.12*
NP-9	0.15	9.0±2.31*

Compared with the group of 1.0% concentration. *P<0.05 and **P<0.01.

Antifertility effects of TPR in rabbit by intravaginal administration

For increasing mating rate, adult female rabbits (n=50) were treated by intramuscular injection with 100 μg of benzestrofol. Different contents of TPR in vaginal suppository (0.0, 0.1, 0.4 and 1.0 g) were put to the vaginal vault as reported (Shrabanti et al., 2007) with a specially made glass tube (n=10, each content). 50 mg NP-9 suppository was applied as positive control. After 15 min, the female rabbits mated with male rabbits only once and the vaginal scraping with plenty of prorsad-mobiling sperm was identified as successful copulation. The litter size and corepus luteum were recorded to calculate the pregnant rate after 15 days.

Culture media and strains

500 g of TM transport medium was contained by 19 g of agar powder, 5 g of haemoglobin and 10 ml of Oxiod Vitox supplements. And the MH transport medium was freshly prepared in our laboratory with 1 g of beef extract, 8.75 g of enzymatic digestion casein and 0.75 g of amylum solubile. The three WHO control strains B (29501), W_1 (29400), W_2 (29403) were dissolved in MH and then cultured on TM medium. All samples against *N. gonorrhoeae* strains were determined by the agar dilution method. Freeze-fried powders of each extract were dissolved after sterile under uviol lamp (Putnam et al., 1992). The solutions of tested extracts were diluted by two fold serial dilution to make a serial of different concentrations. The stock solutions of antimicrobial agents were prepared using autoclaved GC agar base mixed with the Hemoglobin solution, after cooling to 50 centigrade, supplemented with Oxiod microbial agent. In addition, 18 ml of stock solution was combined with TPR solutions together to product a series of agar plates containing different concentrations of tannin. Fresh overnight colonies were suspended and diluted in sterile beef broth to a known density by using Mc Farland standard method. The density is about 10^7 cfu of *Gonorrhoeae neisseria* strains per milliliter. Then, 2 ul of beef broths were inculcated on each agar plates. The plates were incubated for 24 h.

Statistical analysis

The *t*-test was used to determine statistical difference. Values of $P<0.05$ were considered statistically significant.

RESULTS

Phenolics in TPR

The yield of extract obtained from pomegranate rind using ethanol was 17.23% (w/w). The TPR was proved to contain 283 ± 4 mg/g total polyphenolics standardized as equivalent of gallic acid. The polyphenolics in different fractions were 385 ± 7 mg/g (20%), 326 ± 3 mg/g (40%), 269 ± 2 mg/g (60%), 235 ± 2 mg/g (70%), 197 ± 2 mg/g (10%) and 161 ± 8 mg/g (H_2O), respectively.

The spermicidal efficacy of TPR

Pomegranate rinds were found to have the role of agglutination, causing sperm immobilization. When agglutinating, the semen aggregated randomly. The minimum concentration of Pomegranate rinds that caused 100% immobilization within 20 s with no revival of motility at 37℃ was considered to be the minimal effective concentration (MEC). The MEC of Pomegranate rinds was 2.0%. While the concentration was lower than 2.0%, the sperm lost some motility; while the concentration was more than 2.0%, the sperm lost completely motility (Table 1).

The active parts of TPR

After the sperm were mixed with TPR, the microscopy imaging showed that the sperms expanded quassated or head-tail broken and agglutinated. The sperms were active at low TPR concentration, but with the concentration increasing, they became to immobilizate significantly, from active to trembling and lost the activities eventually. Statistical results indicated that the 20% CH_3OH eluting part had the highest inhibiting activity, and the MEC in 20 s was 1.25 mg/ml (Table 2). This eluting part may contain some compounds which have strong inhibition on sperm motility.

Antifertility efficiency in rabbits

All samples in control group were pregnant. The 1.0 g of TPR group was proved to process obviously contraceptive effect. Compared with control group, 100% of contraception was found in the 1.0 g of TPR and NP-9 group. The 0.4 and 0.1 g of TPR group showed low conception rate, 10 and 40%, respectively (Table 3). The minimum inhibitory concentration (MIC) was read as the

Table 2. The effect of several eluting parts of pomegranate rind with different concentrations against the motility of the sperm (n=20).

Drug	mg·ml^{-1}	Action time (s)		
		20	60	180
NP-9	0.50000	-	-	-
	0.25000		-	-
	0.12500	±	-	-
	0.06250	+	+	±
	0.03125	+	+	+
H_2O eluting part	5.00000	-	-	-
	2.50000	±	-	-
	1.25000	±	±	+
	0.62500	+	+	+
	0.31250	+	+	+
10% CH_3OH eluting part	5.00000	-	-	-
	2.50000	±	-	-
	1.25000	±	±	+
	0.62500	+	+	+
	0.31250	+	+	+
20% CH_3OH eluting part	5.00000	-	-	-
	2.50000	-	-	-
	1.25000	-	-	-
	0.62500	±	-	-
	0.31250	+	±	-
40% CH_3OH eluting part	5.00000	-	-	-
	2.50000	-	-	±
	1.25000	±	±	+
	0.62500	+	+	+
	0.31250	+	+	+
60% CH_3OH eluting part	5.00000	-	-	-
	2.50000	-	-	±
	1.25000	±	±	+
	0.62500	+	+	+
	0.31250	+	+	+
70% CH_3COCH_3 eluting part	5.00000	-	-	-
	2.50000	-	-	-
	1.25000	-	±	+
	0.62500	+	+	±
	0.31250	+	+	+
Physiological saline	-	+	+	+

-: Sperm is immobile; ±: 90% semen lost motility; +: sperm is mobile.

Table 3. Effect of CP suppository on antifertility activity in rabbits.

Group	Dose	Pregnant / total rabbits	No. of implantation($\bar{X}$±SD)
Control	-	10/10	4.4±0.79
Pomegranate rinds	0.1 g	4/10	0.9±1.29*
Pomegranate rinds	0.4 g	1/10	0.2±0.63*
Pomegranate rinds	1.0 g	0/10	0**
NP-9	50 mg	0/10	0**

Compared with the control group *P<0.05 and **P<0.01.

Table 4. The minimum inhibitory concentration mg/ml of tannins in *Punica granatum* L. on three kinds of *G. neisseria* strains.

Composition	H_2O Eluting part	10% CH_3OH eluting part	20% CH_3OH eluting part	40% CH_3OH eluting part	60% CH_3OH eluting part	70% CH_3OH eluting part
B (29501)	2.5	1.25	2.5	5.0	5.0	2.5
W1 (29400)	2.5	1.25	1.25	5.0	5.0	2.5
W2,3(29403)	2.5	2.5	1.25	2.5	5.0	2.5

lowest concentration of antibiotic inhibiting visible growth. The results of the antigonorrhoeal assays were recorded in Table 4.

DISCUSSION

In the spermicidal experiments, first pomegranate rind was found to inhibit the sperm motility by following a modified version of the Sander-Cramer test *in vitro*; then the spermicidal part of tannin in pomegranate rind was screened by WHO standard method, and the 20% CH_3OH eluting part had the highest inhibiting activity. A dose- and time-dependent reduction in sperm motility was observed by adding TPR and TPR to semen samples (Tables 1 to 3). All of them were found to inhibit sperm motility, with the concentration increasing or the time prolonging, the inhibition became stronger and stronger. The MEC of NP-9 on inhibiting sperm activity in 20 s is 0.125 $mg \cdot ml^{-1}$, consistent with the reports (Chantler et al., 1992). The eluting parts of tannin in pomegranate rind were found to inhibit the sperm activity significantly and the MEC of the 20% CH_3OH eluting part in 20 s was 1.25 $mg \cdot ml^{-1}$, which was less than that of NP-9. It is feasible to screen a new spermicide from pomegranate rind in further studies. However, the mechanism by which dermaseptin exerts its rapid spermicidal action of pomegranate rind is not known.

The microscopy imaging showed that the sperm quassated or head-tail broken and agglutinated. The phenomenon suggested that tannins from pomegranate rinds may kill the sperm through destructing the substructure and organization of the sperm. Furthermore, tannin-protein reaction indicated that TPR could congeal seminal plasma protein *in vivo* to inhibit mobility of semen, react with sperm membrane protein for decreasing the stability and integrity and deactivate the metabolic enzyme (both the metal center and protein) to interrupt the energy-supplying chain. Further researches would focus on the precise spermicidal mechanism of TPR by transmission electron microscope and concrete process which TPR adheres the spermatic protein (Data not shown). The results of vaginal suppository in rabbits exhibited that when the content of TPR was more than 0.4 g, It showed confirmed forceful antifertility efficiency and when the content is 0.1g, it also occurred the activity to decrease the number of implantation [0.9 ± 1.29, (Table 3)]. The contraceptive mechanism of vaginal suppository in rabbits may be: agglutination of TPR to semen; destructing seminal plasma protein; changing the internal environment which is essential to sperm; dispersing sperm to free state and decreasing the spermatic mobility. Respecting the tolerance of rabbit sperm is double compared with human semen, so it seems that satisfied effects may be observed in clinical trail.

Gonorrhoea is a sexually transmitted disease which is caused by the bacteria, *N. gonorrhoeae.* So far, appropriate the clinical treatment of gonorrhea mainly uses antibiotics. But disease control is also problematic, because of high rates of changes by way of both spontaneous mutation and misuse of antibiotics in settings (Simonsen et al., 2004). The data from recent report showed that by now in some cities, more than 81.7% of all *N. gonorrhoeae* isolates were resistant to Penicillin G. The increase has been occurring rising trends of gonococcal resistance with the third-generation cephalosporin and fluoroquinolones (Tapsall, 2005). As is reported that, the tannin in pomegranate rind can not only inhibit the sperm motility, but also has the antigonorrhoeal effects. So it is feasible to develop TPR as a new vaginal

contraceptive, which has the role of spermicidal and antibacterial agents.

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