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# Study on Micro-molecular Compounds with SOD-like activity in *Rosa Roxburghii* Tratt

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***Rosa Roxburghii* Tratt,** growing on the plateau mountain areas of southwestern China, is a unique wild plant of China. Usually two meters high, it belongs to a deciduous shrub family. Its fruit, with a flat round shape, is yellow when ripe with soft thorns on rind. The pulp, full and fleshy, sour and sweet, contains more than 35 kinds of nutrients[1]. It has a rich fragrance and has more than 130 kinds of volatile fragrances[2]. The fruit can be eaten raw. Growing in a wild environment and being free from pesticide pollution, it is a very rare wild plant source. In 1987 and 1988, Wuli reported[3-6] that the fruit of *Rosa Roxburghii* Tratt contains an abundance of Copper and Zinc based Superoxide Dismutase (SOD) with molecular weight of 34,000 daltons. In 1992, with a more advanced technique, the author isolated the pure SOD[7], thus making industrial mass-production possible. SOD is the only enzyme that can catalyze superoxide anion dismutation. McCord and Fridovich were the first to create the Xanthine Oxidase-Cytochrone classic C method to determine activity of SOD[8]. After that, the XOD-NBT reduction method[9] , classical pyrogallol autoxidation method[10], chemiluminescence method[11] and micro-pyrogallol autoxidation method [12] etc. are reported.

We determined the activity of SOD in *Rosa Roxburghii Tratt* in accordance with the method as defined in document [12], and confirmed that the total SOD activity of 100g *Rosa Roxbrughii Tratt* fruit is 217,982 units. In contrast, the method as defined in document [7], state that only 29,800 units pure macro-molecular SOD can be extracted from 100g fruit. Obviously, besides macro-molecular SOD, the fruit also has other compounds capable of scavenging superoxide free radical activity. These compounds are called SOD-like compounds because they have SOD-like activity.

**This paper is the first one to report on these SOD-like compounds in Rosa Roxburghii Tratt and some of their characters.**

## I. Materials and instrumentation

### 1. Materials

a. Fresh *Rosa Roxburghii* Tratt fruit, collected from Leye county, Guangxi, Peoples Republic of China
b. Preparation: extract the fruit in mill engine, after high-speed centrifugation, take the upper clear liquid.
c. Standard sample: SOD, Vitamin C and Vitamin E, produced by Sigma Co., U.S.A.
d. Euscaphic acid, Tormentic acid and Roxburic acid, isolated and purified by the author from the fruit and identified by IR Spectrophotometer.
e. Chemical reagents are all analytically pure.

### 2. Instrumentation

a. Beckman DU-7 UV--Vis Spectrophotometer, made in U.S.A.
b. Waters 201DC High performance Liquid Chromatograph, made in U.S.A.
c. Shimadzu GC-9A Gas Chromatograph, made in Japan,
d. LKB Automatic Column Chromatograph System, made in Sweden
e. Nicolet 5DXB Flouriev Transform Infrared Spectrophotometer, made in U.S.A.
f. Fluorescence Spectrophotometer, made in Hitachi, Japan**.**

## II. Experimental methods:

1. Isolation and purification of SOD in *Rosa Roxburghii* Tratt are performed in accordance with the methods as defined in document [7].
2. **Confirmation and determination of SOD and SOD-like activity:** The XOD- Cytochrone C method as defined in document[8] and micro-pyrogallol autoxidation method as defined in document[12] are used to confirm the SOD activity
3. **Determination of Vitamin C and Vitamin E content in *Rosa Roxburghii* Tratt:**The 2,4-dinitrophenylhydrazine method [13] is used for determination of Vitamin C and HPLC method [14] for Vitamin E
4. **Extraction of triterpene acid mixture in *Rosa Roxburghii* Tratt.**

While using method in document [15-16], we made improvements to the experiment by extracting the mixture only. We choose 1000g raw fruit powder of Rosa Roxburghii Tratt. combined with 95% ethanol reflux to extract, under reduced pressure, after concentration. A cream paste was formed. We added water, used petroleum ether to extract, got rid of impurities, used ethoxyethane to extract aqueous phase, recovered ethexyethane and got 12g outcome A; use n-butanol to extract aqueous phase again, 45g outcome B is obtained. Combining A and B, We used silica gel column chromatography, dilute with $CHCl_3$-MeOH(20:2), collected fractionally, used silica gel TLC, developed with MeOH-$CHCl_3$(2:20), and coloured with 5% PMA ethanol solution, combine dilution liquid with $R_f$=0.43-0.49, and concentrated it. After heating the solution with MeOH, precipitation appeared and the upper clear liquid was taken. Under reduced pressure, it is dried by distillation to triterpene acid mixture. Because of their similar structures, we used HPLC instead of common column chromatography to isolate and purify the mixture.

5. **HPLC isolation and determination of triterpene acid:** Triterpene acid mixture, which has been isolated with silical gel column chromatography is solveded with methanol. On μ Bondapar $C_{18}$ column, mobile phase is made with MeOH-$H_2O$ (1:9), and detection is made on 254 nm. After appraising acid time with standard Euscaphic acid, Tormentic acid and Roxburic acid, quantitative analysis is made based on peak area.
6. **Experiment on heat stability of SOD and SOD-like compounds in *Rosa Roxburghii* Tratt:** Macro-molecular SOD, Vitamin C + E, Triterpene acid mixture solution are compounded referring to their content in *Rosa Roxburghii* Tratt juice, and together with the fruit juice put into 75°c and 100°c water bath for 20 minutes. The SOD and SOD-like activity before and after the treatment are determined.
7. **mp. $?_{max}$ and IR $?_{max}$ of three kinds of triterpene acids are determined with instrument.**
8. **Animal experiments**
    a. **Experiment on a rat's resistance to lack of oxygen when decompressed**: 30 rats weighing 18 to 23g were chosen and randomly divide into three groups with 10 in each group. The first group of rats were abdominal injected with 20 ml mixture of 4.25mg/ml triterpene acid and 34.98mgVitC/ml; the second group with 20ml *Rosa roxburghii* Tratt juice and the third group with 20ml normal saline water. Forty minutes later, two rats of each group were placed in a 250ml wide-neck flask and decompressed (530 mmHg). Their survival times were observed.
    b. **Experiment on rat's resistance to fatigue (swimming time):** 30 rats weighing 18 to 20g were chosen and randomly divided into three groups. Rats in the first and second groups were orally injected for seven consecutive days with*Rosa Roxburghii* Tratt juice and micro-molecular SOD solution 0.2ml /10g / weight respectively. The control group was orally injected with equal amounts of normal saline for seven consecutive days, and once two hours before the experiment. The rats were placed into a 40cm deep plastic bowl at 30±1°C constant temperature, swimming times were recorded until they sank and died.

c. **Experiment on effect on lipofuscine in rat:** 12 healthy rats weighing 350 ± 50g. were selected. An experimental model of subacute senility induced by d-galactose is established in rats according to Xu's method. The rats were divided into three groups. The first group - senility mode group. We orally injected normal saline 0.5ml/100g daily. The second group, triterpene acid +Vitamin C, Vitamin E group, are orally injected with 0.5ml/100g, equal to content of *Rosa Roxburghii* Tratt juice. The third group, *Rosa Roxburghii* Tratt juice group are orally injected with ginseng extract 0.5ml/100g, equal to 4.0mg ginseng. Thirty-two days later, the rats were dissected, 1g of heart, liver and brain tissue on two sides were taken. After washing away blood and drying with filter paper, Each rat was weighed, placed into a test-tube with plugs. The lipofuscine effect on rats were determined by fluorimetry as per document [18].

9. **Test of anti-lipoid peroxidation of human being:** All samples were collected from volunteers with age between 50 and 75 years old. They had an average age of 58.1. A total of 52 males and 48 females volunteered to accept the test. Each volunteer drank 80 ml Qi Gong Jian oral solution made of the Rosa Roxburghii juice, twice daily, for a two-month period. Blood samples were taken in the morning before breakfast. The SOD activity of erythrocytes was detected using pyrogallol autoxidation method[12]and the lipoid of blood plasma was detected by fluorescence method[19]. The concentration of haemoglobin was determined by the Kulter's haemacytometry method.

## III. Results and discussion.

1. SOD activity in *Rosa Roxburghii* Tratt can be divided into macro-molecular and micro-molecular SOD activity. The Micro-pyrogallol autoxidation method can directly determine SOD activity in the juice, and that is total activity. When the juice is placed into a dialyzator and dialyzed with 0.05mol phosphate buffer solution (PH 7.8), the activity is determined after removing the micro-molecular compound with SOD-like activity, and that is the activity of macromolecular SOD. The difference between these two is micro-molecular SOD-like activity, as in table 1:

**Table 1**
Macromolecular SOD and micro-molecular SOD activity in *Rosa Roxburghii* Tratt (RRT)

| Content | Total activity of RRT | Macromolecular SOD | Micro-molecular SOD |
|---|---|---|---|
| | RRT (before dialysis) | (after dialysis) | (before-after dialysis) |
| Activity (unit/ml) | 4359.64 | 579.83 | 3779.81 |
| Percentage | 100.00 | 13.30 | 86.70 |

The table shows that besides the well-known macro-molecular SOD, there is a micro-molecular SOD-like compound, which can pass the dialysis membrane.

2. **What are those micro-molecular compounds with SOD-like activity?** Among more than 35 nutrients which have been found in *Rosa Roxburghii* Tratt in document[14], only Vitamin C, Vitamin E and Triterpene acid compounds have SOD-like activity after determination by the micro-pyrogallol autoxidation method. Results of using XOD-Cytochrone C, are the same as above. As in table 2, no SOD activity can be detected from compounds such as amino acid, tannic acid, citric acid and malic acid.

**Table 2**
Micro-molecular compounds with SOD-like activity in *Rosa Roxburghii* Tratt

| Composition | Vit C | Vit E | Triterpene acid mixture ( crude extract ) |
|---|---|---|---|
| Concentration (mg/ml) | 2.12 | 2.02 | 2.05 |

| | | | |
|---|---|---|---|
| Activity by p-a method (unit/ml) | 142.04 | 20.36 | 713.07 |
| Activity by XOD-Cychrone(unit/ml) | 97.45 | 14.05 | 481.82 |

Note: Because the activity definition of pyrogallol autoxidation (p-a) method and XOD-Cytochrone C are different, the data are different, too.

3. **Determination of content of micro-molecular SOD-like compound in *Rosa Roxburghii* Tratt and inferring its activity in 100g fruit:** The content of Vitamin C and Vitamin E in *Rosa Roxburghii* Tratt were determined by normal chemical and instrumental analysis. In respect of triterpene acid, a newly found compound, as per document[15], only a qualitative test and not quantitative analysis were performed. Based on column chromatography, the HPLC were used to determine a quantitative analysis. The pyrogallol autoxidation method were used to determine SOD-like activity of each compound. The fruit's activity can be inferred as in table 3.

**Table 3**
Content of Vit C , Vit E and Triterpene acid in *Rosa roxburghii* Tratt

| | Vit C | Vit E | Triterpene acids | | |
|---|---|---|---|---|---|
| | | | Euscaphic acid | Tormentic acid | Roxburic acid |
| Content (mg) | 1747.57 | 2.56 | 90.02 | 6.73 | 4.28 |
| SOD-like Activity(unit/mg) | 67 | 10.24 | 702.58 | 704.81 | 906.77 |
| Activity in *RosaRoxburghii* Tratt (unit/100g) | 117087.2 | 26.21 | 63246.2 | 4743.4 | 3881.0 |

4. **Heat stability test of each SOD-like compound in *Rosa Roxburghii* Tratt :**Macro-molecular SOD, Vitamin C + Vitamin E , Triterpene acid mixture and *Rosa Roxburghii* Tratt juice were placed into a 75°C and 100°C water bath for 20 minutes. Before and after treatment activity results can be seen in table 4.

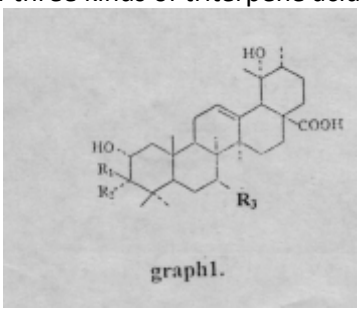
**Table 4**
Heat stability of SOD and SOD-like compounds

| Composition | Rosa juice | Macro-molecular SOD | VitC+VitE | Triterpene acid |
|---|---|---|---|---|
| Concentration(mg/ml) | (original juice) | | 34.98 | 4.25 |
| Activity before Teatment(unit/ml) | 4359.64 (100%) | 579.8 (100%) | 2342.27 (100%) | 1435.21 (100%) |
| Activity after 20 minutes 70°C treatment(unit/ml) | 4180.90 (95.90%) | 481.95 (83.12%) | 2164.02 (92.39%) | 1535.67 (107.00%) |

| | | | | |
|---|---|---|---|---|
| Activity after 20 minutes 100°C treatment(unit/ml) | 3684.33(84.51%) | 26.10 (4.5%) | 2087.90 (89.14%) | 1548.59 (107.90%) |

Note: Numbers in brackets are the remaining activity percentage after the heating treatment.
Table 4 shows that the heat stability of macromolecular SOD is quite weak, with only 4.5% activity left after 100°C water bath treatment. The activity of Vitamin C and Vitamin E is higher, with 92.39% left after 75°C and 89.14% after 100°C water bath treatment. The activity of triterpene acid increased, instead of decreased. This is due to the existence of triterpenoid saponins [17] in the fruit, which is partly hydrolyzed to triterpene acid in an acid condition after heating.

5. **Structure and physical chemical nature of three kinds of triterpene acids:** Liang guangyi reports [15-16] the structures of three kinds of triterpene acids are as shown in graph 1.



graph1.

**$R_1$ $R_2$ $R_3$ $R_3$**
**euscaphic acid H OH H**
**tormentic acid OH H H**
**Roxburic acid H OH OH**

Among them euscaphic acid and tormentic acid are a couple of epimers, with a difference in $C_3$ hydroxy only. The former is a a-configuration and the latter a ß-configuration. Both are derivatives of ursolic acid. Roxburic acid was recently found, with structure 2 ß ,3 a ,7 ß,19 a -tetrahydroxyurs-12-en-28-oic aid. Some of the physical and chemical natures are shown in table 5.

**Table 5**
Physical chemical nature of three kinds of triterpene acids

| acid | mp | M.F | M.W | $?_{max,nm}$ | IR (?,$cm^{-1}$) |
|---|---|---|---|---|---|
| Euscaphic acid | 264~226 (AcOEt) | $C_{30}H_{48}O_5$ | 488 | 202 | 3700~2400 |
| Tormentic acid | 264~267 (MeOH) | $C_{30}H_{48}O_5$ | 488 | 205 | 3700~2400 |
| Roxburic acid | 292 (lytic) | $C_{30}H_{48}O_6$ | 504 | 206 | 3650~2400 1685,931 |

From graph 1 and table 5, we can see that the three triterpene acids share a common character: ursane configuration of 30 carbon atoms, 3-4 hydroxies, one double bond, a carboxy on $C_{12}$,and on $C_{28}$, the molecular weight is much smaller than SOD. , The acids are very easily absorbed in human body.

6. **Results of animal experiments are in table 6,7,8.**

**Table 6**
Experimental results of rat's resistance to lack of oxygen when decompressed

| Group | Number Of Rats | Survival time | | P (compare with Control group) |
|---|---|---|---|---|
| | | X±SD(min) | Survive % | |
| Control | 10 | 3.8±3.5 | / | / |
| Juice | 10 | 8.9±6.1 | 134.2 | <0.05 |
| Micromolecular SOD | 10 | 7.9±3.7 | 107.9 | <0.05 |

It shows that the micro-molecular SOD found in *Rosa Roxburghii Tratt* juice can obviously prolong the survival time ($p<0.05$).

**Table 7**
Experimental results of rat's swimming time

| Group | Number of rats | Swimming | P Value |
|---|---|---|---|
| | | Time | Compare with |
| | | X±SD(min) | control group |
| Control | 10 | 90.15±30.56 | |
| Juice | 10 | 189.32±5.91 | <0.01 |
| Micromolecular sod | 10 | 172.48±4.12 | <0.01 |

It shows that micro-molecular SOD found in the *Rosa Roxburghii* Tratt juice can obviously prolong swimming time ($p<0.01$),which means that both can build up health and have good effect of anti-fatigue.

**Table8**
Lipofuscine in rat's tissue.

| Group | Number of rat | Lipofuscine(u/g) | | |
|---|---|---|---|---|
| | | Heart | Liver | Brain |
| Senility mode | 4 | 42.±11.95 | 53.1±16.8 | 33.15±12.86 |
| Juice | 4 | 24.55±4.67 | 27.8±6.99 | 13.69±2.99 |
| Micromolecular SOD | 4 | 25.02±5.23 | 27.9±7.02 | 15.16±3.51 |

(Note: In each group, p<0.01)

**It shows that compared with senility mode group, both *Rosa Roxburghii* Tratt micromolecular SOD and juice can reduce lipofuscine in body tissue, which means that both can scavenge superoxide free radicals in the body.**

7. **Effect of anti-lipoid peroxidization in the human body:** The changes of lipoid in blood plasma, SOD activity of erythrocytes and the rate of SOD/Lipoid (LPO) shown in Table 9 to 11 following, pre- and post oral administration of the Qi Gong Jian oral solution.

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**Table 9**
The concentration changes of LPO in blood plasma following pre- and post oral administration

| Sex | No. Of human | LPO( n mol/ml) | | |
|---|---|---|---|---|
| | | Pre-oral | Post oral | |
| | | X ± SD | X ± SD | P Value |
| Male | 52 | 4.25 ± 0.78 | 3.41±0.62 | <0.001 |
| Female | 48 | 4.65 ± 0.42 | 3.63±0.47 | <0.001 |
| Total | 100 | 4.45 ± 0.63 | 3.53±0.56 | <0.001 |

**Table 10**
The changes of SOD activity erythrocytes - pre- and post oral administration.

| Sex | No. Of human | SOD activity (u/g Hb) | | |
|---|---|---|---|---|
| | | Pre-oral | Post oral | |
| | | X ± SD | X ± SD | P Value |
| Male | 52 | 1538.25±520.32 | 2526.3±434.32 | <0.001 |
| Female | 48 | 1581.37±586.25 | 2638.2±516.22 | <0.001 |
| Total | 100 | 1559.86±553.29 | 2583.3±475.31 | <0.001 |

**Table 11**
Comparison of the rates of SOD/LPO pre- and post oral administration.

| Sex | No. Of | SOD activity (u/g Hb) |
|---|---|---|

| | human | Pre-oral | Post oral | |
|---|---|---|---|---|
| | | X ± SD | X ± SD | P Value |
| Male | 52 | 358.25±86.71 | 750.22±78.42 | <0.001 |
| Female | 48 | 336.15±76.32 | 685.50±72.59 | <0.001 |
| Total | 100 | 367.72±98.45 | 731.61±78.13 | <0.001 |

As a result, when humans periodically consume *Rosa Roxburghii Tratt* juice as a oral solution made of pure Rosa Roxburghii Tratt fruit juice, the LPO concentration of blood plasma decreases, SOD activity of erythrocytes increases and the rates of SOD/LPO increases. It therefore indicates the ability to increases the anti-lipoid peroxidation in the body.

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## IV. Conclusion

Besides macromolecular SOD, *Rosa Roxburghii* Tratt also contains natural compounds with SOD-like activity, including Vitamin C, Vitamin E and some triterpene acids. Because of their small molecules, they can be easily absorbed in the human body. They are capable of scavenging superoxide free radicals in the human and animal body. Due to excellent heat stability, these elements don't easily lose their stability during product processing.

These compounds have a very good commercial and clinical value. When human beings periodically drink*Rosa Roxburghii Tratt* fruit juice oral solution made of pure Rosa Roxburghii Tratt fruit, LPO concentration of blood plasma decreased remarkable, The SOD activity of erythrocytes increases. Therefore the higher the rate of increase in SOD erythrocytes the more rapid the reduction in LPO. It indicates that the ability of anti-lipoid peroxidation strengthenes the body.

Potchefstroom University Clinical Trial

Dr. Lardus Erasmus (promoter), Dr. F van der Westhuizen (Bio Chemist)

Gerhard Joubert –Life Enrichment Institue - Owner of research

**Cili Bao - Superoxide Dismutase Preliminary Project**

(for The Life Enrichment Institute - Gerhard Joubert)

**Truncated protocol**

*Subjects:*

16 (8 men / 8 women) *normal group*

8 HIV group

8 Diabetic group

*Products:*

Cili bao fruit extract / pulp Supplement 1

Cili bao fruit extract / pulp + Chlorella Supplement 2

Control group (placebo)

*Administering protocol:*

**A Potchefstroom Group (normal group)**

- 1 serving = 10 ml (10 mg)
- All subjects are monitored for 2 weeks prior to supplement intake and 2 weeks after supplement intake duration.
- Diet of subjects are monitored.
- 4 persons taking 1 serving of Supplement 1 with "Endurance" breakfast, lunch and dinner. Daily for 6 weeks.
- 4 persons taking 1 serving of Supplement 2 with "Endurance" breakfast, lunch and dinner. Daily for 6 weeks.
- 4 persons taking 1 serving of Supplement 3 with "Endurance" breakfast, lunch and dinner. Daily for 6 weeks.
- 4 persons taking no additional supplements would serve as controls.

B **HIV group**

Administering of supplements will be similar to Group A.

C **Diabetic group**

Administering of supplements will be similar to Group A.

*Analysis*

A blood sample (5 ml) of each of the subjects where taken once a week and the following parameters measured:

1. SOD in plasma and erythrocytes
2. Hemoglobin/oxyhemoglobin/methemoglobin ratio
3. Serum Total peroxyl radical-trapping potential (TRAP)
4. Reduced Gluthatione
5. Cyto-toxicity
6. LDL and HDL
7. CD4 and CD8
8. Lymphocytes – full spectrum