Markhamia tomentosa enhanced the antimalarial activity of chloroquine and amodiaquine in chloroquine resistant P. berghei infected mice

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ABSTRACT

The prevalence of malaria is on a steady increase in Nigeria. However, people residing in the areas of high malaria transmission use medicinal plants in combination with standard antimalarial drugs. This study aimed to investigate the effect of aqueous leaf extract of Markhamia tomentosa (Benth.) K. Schum. Ex Engl. (Bignoniaceae) on the antimalarial activity of standard antimalarial drugs using Peters’s 4-day suppressive test. Also, identify the chemical constituents of n-hexane fraction of the plant using GC-MS spectrometry was identified. Standard antimalarial drugs [chloroquine, amodiaquine (10 mg/kg/day) and artesunate/amodiaquine (4/10 mg/kg)] were combined with 100, 250 and 500 mg/kg/day extract of M. tomentosa once daily for 3 days using a 4-day suppressive test. Survival of animals were assessed till day 21 (D 21). Combination of amodiaquine (AQ) and M. tomentosa or artesunate amodiaquine (AS/AQ) and M. tomentosa (100-500 mg/kg) significantly reduced chemosuppression of parasite growth. On D 14 p.i. chemosuppression of parasite growth in infected mice treated with the combination of amodiaquine (AQ) and M. tomentosa group was significantly (p < 0.05) higher (78-88%) than mice treated with AQ alone (67%). In addition, combination of chloroquine and MT at 200 or 500 mg/kg (71.09 - 91.66%) produced significant chemosuppression of parasite growth than chloroquine (CQ) alone (0.0 – 19.06%) on day 12 and 14. Likewise higher survival of experimental mice was observed in CQ + 500 mg/kg MT than animals treated with CQ alone. Analysis of the n-hexane fraction with GC-MS revealed fifteen constituents, Heptaedecane and Hexadecane were the most prominent (20.49% and 35.55%) respectively. M. tomentosa augments the antimalarial activity of chloroquine and amodiaquine in mice infected with chloroquine-resistant P. berghei ANKA strain.

Keywords: Ethnomedicine, Markhamia tomentosa, Antiplasmodial, Standard drugs, Chemosuppression, and Gas Chromatography and Mass Spectrometry.

INTRODUCTION

Malaria remains a public health challenge. A clinical report of the World Health Organization, revealed 214 million cases of malaria globally in 2015. Malaria has led to death of both expectant mothers and toddlers. Rates of disease have decreased from 2000 to 2015 by 37% [1]. Also, the increasing prevalence of malaria parasites, Plasmodium falciparum resistance to most of the orthodox drugs pose an imminent danger to the management of malaria [2], The resistance is not limited to artesmins-based combination therapy (ACT) and this has called for deep concern in the treatment of malaria [3]. Several reports have shown that due to poor economic situation and insufficient health facilities among the people in malaria prevalent clime, simultaneous use of herbal remedies with orthodox antimalarial drugs is a common practice [4-7]. Scientific validation of such practice is important to establish new fact of the native.

The efficacy of traditional systems of medicine largely depend on the use of suitable plant part, the nature of secondary metabolite, application and dosage of crude drug, and the duration of the treatment [8-10]. There is an uprising comparative information on the bioactive molecules of a medicinal plant with its pharmacological usage. Gas Chromatography Mass Spectroscopy is a compound technique, commonly used in the identification and quantification of bioactive constituents of a medicinal plant.

Markhamia tomentosa (Benth.) K. Schum. Ex Engl. (Bignoniaceae) is a tree of about 15-m high and is found largely in Africa around the Savannah and rain forests region. The local (Yoruba, Nigeria) names are Akoko, Oruru [11]. The leaves and stem bark are used to treat different ailments like oedema, rheumatoit, arthritis, gout, and muscular pain in Nigeria [12, 13]. Various extracts of M. tomentosa have been described to possess several remedial properties, such as antimicrobial, antioxidant, antiulcer, and...
analgesic properties [14,17]. The leaf extract has been reported to exhibit antimalarial potentials [18] which supported their traditional medicine to treat fevers [11, 19-20].

This study investigates the in vivo antimalarial activities of aqueous leaf extract of the plant alone and in combination with three readily obtainable anti-malarial drugs (chloroquine (CQ), amodiaquine (AQ) and Aternsate amodiaquine (AS/AQ)) in a mouse model of P. berghei and the identification of the chemical constituents of the plant.

MATERIALS AND METHODS

Plant collection and authentication

Fresh leaves of *M. tomentosa* were collected from Oke-Igbo (7.9736°N, 4.4415°E) in Ondo State, Nigeria in September, 2015. Plant was identified and authenticated in the Herbarium of the Department of Botany, University of Lagos with a voucher specimens’ number LUH 3936.

Plant extraction

The leaves of *M. tomentosa* was hot dried in an air oven at temperature (30 ± 0.5 °C) and pulverized in a mechanical grinder to a coarse powder. The plant parts (1 kg) was extracted in 4 L of water in compliance with traditional preparation for 40 minutes. The resulting extract was cooled and filtered. It was then freeze dried, and the resulting extracts were stored at 4 °C. The extractive yield was 20.1% (w/w). Extraction process was repeated and the yield crude extract (19.66%) (w/w) was dissolved in distilled water and partitioned in the order of *n*-hexane, dichloromethane, ethyl acetate, butanol and water. All fractions were concentrated using rotary evaporator at 40°C. The fraction gave the resulting yield of 0.2ml, 1.96g, 0.64g, 0.23g and 16.26g respectively.

Experimental animals

In vivo antimalarial activity of plant extract used eightye mice (7-10 weeks old, weighing 20 ± 2 g) of either sex were procured in the Nigeria Institute of Medical Research, Lagos State, Nigeria. They were preserved in separate cages at 22 °C under natural 12 h day/ night conditions in the animal houses of the Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Oyo State, Nigeria and maintained in specific pathogen-free animal facility. The animals were preserved under strict laboratory rules, as approved by the Experimentation Ethics Committee of the College of Medicine, University of Lagos (CM/COM/08/VOL.XXVI) which supported our project. The animals, acclimatized for 12 days preceding the experiments, were fed with ACE Feeds PLC, Ibadan, Oyo State, Nigeria) and had drinking water *ad libitum*.

Malaria parasite

Chloroquine-resistant *P. berghei* ANKA strain used for the study was obtained from Medical Molecular Biology Research Unit, National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand. Each donor mouse parasitemia level was determined under microscopically giemsa-stained thin blood smears and serial dilution of blood with normal saline which was used as downwards adjustment. A standard inoculum of 1 × 10⁷parasitized erythrocytes was administered (200 ml) to each mouse intravenously at the tail region. The day one of infection was marked as day zero (D 0) and D 1, D 2, etc for the following days of the experiment. Eighty mice were divided into sixteen groups, with each group having 5 mice after infection with 1 × 10⁷ parasitized erythrocytes ANKA strain. All experimental groups were set at the same time. The animals were treated with the aqueous extract of *M. tomentosa* (100, 250 and 500 mg/kg) alone and/or in combination with antimalarial drugs, chloroquine (10 mg/kg), amodiaquine (10 mg/kg), or amodiaquine/artesunate (4/10 mg/kg). A group test mouse received distilled water (served as negative control). Treatment with antimalarial drugs and extracts started three hours post infection and were given once daily through oral route. Antimalarial drugs and extracts were given for three and four days respectively (D 2 p.i. and D 3 p.i.) respectively [21-22]. Determination of parasitemia was done daily under the microscope of Giemsa- stained thin blood smears prepared from experimental mice on the third day p.i.

Survival time

Monitoring of the mortality rate was done daily at the beginning of drug administration (days 7, 14 and 21) in order to evaluate the percentage survival of the tested mice in all the groups.

Gas Chromatography and Mass Spectrometry of *Markhamia tomentosa*

Fixed oil of *M. tomentosa* was obtained from the hexane fraction. GC-MS analyses was carried out using Agilent technologies 5975C mass spectrometer interfaced with an Agilent technologies 7800A gas chromatograph with an HP5 column (30 m × 0.32 mm id, 0.25 μm film thickness) and an MS detector. The oven temperature was adjusted from 80°C (after 2 minutes) to 220°C at 4.5 °C/min, final temperature was held for 2 minutes at 220°C. The ion source was fixed at 240°C and electron ionization at 70 Ev. Helium was used as the carrier gas (1 ml/min). The split ratio was 1:25 with the scan range of 35 to 425 amu. Hexane fraction (1.0 μL), diluted in hexane was manually injected into the GC/MS. The spectrums of the components identified were compared with the spectrum of known components in the GC-MS library database. The calculation for the percentage composition per component identified was from the addition of the peak areas of the total oil composition. The Kovat index value was calculated for the GC-MS analysis of the plant.

\[
K_I = \frac{100 \left( T_A - T_Z \right)}{T_Z \left( T_A - T_Z \right)} + 100
\]

\[T_A = \text{Your compound peak Retention Time (RT)}\]

\[T_Z = \text{RT of peak before compound peak for alkane}\]

\[T_{Z+1} = \text{RT of peak after compound peak for alkane}\]

Statistical analysis

Percentage chemosuppression of each drug/combination was calculated using this formula: Chemosuppression of parasite growth \[= 100 - \left( \frac{\text{mean parasitemia treated/mean parasitemia control} \times 100}{} \right)\]. Analysis of variance between groups (ANOVA) was used to compare difference in percentage suppression of parasite growth in the treatment and control groups (GraphPad® 5). Chi-square with Yates correlation was used in analyzing the proportions of survival. *p*-value < 0.05 was considered significant

RESULTS

The interaction between *Markhamia tomentosa* (MT) and amodiaquine (AQ), artesunate/amodiaquine (AS/AQ) and chloroquine (CQ) was evaluated using percentage chemosuppression of parasite growth in all the treated groups and percentage survival of the experimental animals. In our previous study on the different doses (250, 500 and 800 mg/kg) of *Markhamia tomentosa* (MT), we reported that MT possessed antimalarial activity. Thus, this study evaluates the potential beneficial interactions between MT and standard drugs. The percentage parasitemia in mice treated with extract of *Markhamia tomentosa* (MT) alone or in combination with standard dose of amodiaquine (AQ) or artesunate/amodiaquine (AS/AQ) is presented on table 1.

Percentage parasitemia in AQ alone or in combination with *Markhamia tomentosa* (MT) 100 - 500 mg/kg ranged from 0.0 to 6.1%, 0.5 to 7.2% on D 4 – D 21 p.i. (table 1). Also, percentage parasitemia in mice treated with standard dose of AS/AQ alone or in combination with *Markhamia tomentosa* (MT) 100-500 mg/kg ranged from 0.0% to 3.6%, 0.4% to
The Journal of Phytopharmacology

2.9% respectively on D 4 – D 21 p.i. (table 1). Parasitemia in the negative control ranged from 8.3 to 18.1% on D 4 - D 14 p.i. Furthermore, the parasitemia of mice treated with CQ alone or in combination with Markhamia tomentosa (MT) 100 - 500 mg/kg ranged from 0.0 to 12.7%, 2.3 to 12.2%, respectively on D 4 – D 21 p.i. (table 1).

Chemosuppression effect of MT in combination with amodiaquine (AQ) on parasite growth

The effect of AQ alone or in combination with Markhamia tomentosa (MT) are presented on table 2. The percentage chemosuppression of parasite growth in AQ alone or AQ+MT from D 5 to D 13 were similar. The same applies to the survival rates of the tested mice in AQ alone of AQ+MT groups (table 3). However, by D 14, the chemosuppression activity of the combinations of MT and AQ was significantly (p < 0.05) higher than that of AQ alone (table 2).

Chemosuppression effect of MT in combination with artesunate/amodiaquine (ASA/Q) on parasite growth

The chemosuppression of parasite growth in mice that received ASAQ alone was 100% while it ranged from 84-85% in ASAQ combined with MT at D 4pi. Even other days there was no significant difference in all the activities of ASAQ alone or in combination with MT (table 2). Likewise, mortality rate in mice treated with combinations with ASAQ or MT combined with ASAQ were similar (table 3).

Chemosuppression Effect of MT in combination with Chloroquine (CQ) on parasite growth

The treatment outcome in mice treated with chloroquine combined with selected doses of M. tomentosa showed delayed activity at the onset of the treatment unlike CQ alone which showed 100% chemosuppression of parasite growth (table 1). From day 7-11, parasite cleared completely in animals treated with chloroquine combined with 250 mg/kg and 500mg/kg of M. tomentosa (table 1). Despite the initial 100% chemosuppression in mice treated with CQ alone, by day 12, percentage chemosuppression dwindled to 19% while it was between 71 – 76% in mice treated with CQ + 250 mg/kg or 500 mg/kg MT (table 2). Also, only treatment with CQ + 500 mg/kg MT produced 40% survival in mice by D 21 p.i. There was no survival in chloroquine alone group, CQ and MT 100 or 250 mg/kg and negative control groups by day 21 (Table 3).

GC-MS result of N-hexane fraction of Markhamia tomentosa

The result of the GC-MS of the n-Hexane fraction showing the chemical composition of the fixed oil of Markhamia tomentosa is summarized in Table 3. Calculation of each kovat index was carried out. Hexadecane has the highest percentage composition of 35.55 followed by Heptadecane 20.49 and Octadecane is 10.9. The least composition of 0.93 was observed in Phenanthrene, 3methyl and 1H-cyclopropa(1) Phenanthrene, 1a, 9b-di hydroy. The GC-MS specimen of the compounds identified is shown in Figure 4.

DISCUSSION

Markhamia tomentosa has been reported for the treatment of fever [11, 20, 24] also both in vitro and in vivo antimalarial activity of the plant had been evaluated [33, 38]. It was observed in this study that the leaf extract of Markhamia tomentosa did not antagonize the antimalarial activity of the standard drugs evaluated. The combination of AQ + MT 100 mg/kg compared to AQ alone exhibited significant chemosuppression of parasite growth at D14. The interaction between AQ and MT appears to be interesting at lower dose of MT. Previous interactions between amodiaquine and another plant (Uvaria camarae) extract at lower doses has been documented [25]. In addition, a 4-fold chemosuppression of parasite growth was observed in mice treated with CQ + 250 or 500 mg/kg of M. tomentosa. In this study there was no survival observed in chloroquine alone treated group, treatment with CQ + 500 mg/kg MT produced 40% survival in mice by D 21 p.i. group. Thus, it appears MT augments the antimalarial activity of chloroquine against resistant parasite.

An earlier report has shown that the leaf extract of Markhamia tomentosa does not exhibit acute or chronic toxicity in rats [27]. M. tomentosa possesses several secondary metabolites including alkaloids, flavonoids that could contribute to the antimalarial properties found in the plant [28, 33]. Several reports have revealed that combination of herbal medicine and / or crude extracts with standard antimalarial drugs have desirable antimalarial activities [29-32]. Large population in some indigenous African community believe in using orthodox antimalarial drugs with medicinal plant preparations [33], they display these practices as a result of poor socio-economic status and insufficient health services in their area.

A combination of an appropriately-dosed crude extracts that contains antimalarial bioactive compounds with a less priced orthodox antimalarial drugs may greatly facilitate elimination of the malaria parasite. This, however provides scientific evidence in supporting their ethnomedical usage. A previous study [38] showed the presence of some phytochemical constituents of the plant which could suggest the possession of potential anti-inflammatory, antimicrobial and antioxidant properties as this may lead to new drug discovery.

Most hexane fractions contain essential oils and some fixed oil. Numerous reports have shown that essential oils and fixed oil possess therapeutic properties which are well tolerated [34-36]. The study revealed that the fixed oil of hexane fractions demonstrated a high level of chemosuppression of parasite multiplication at day 4 (data not shown). The activity of hexane fraction of M. tomentosa is probably due to the presence of hydrocarbons identified by GC-MS in the study. The hydrophilic character of monoterpenes functional groups and the lipophilic character of their hydrocarbon skeleton are of main importance in the antimicrobial action of essential and fixed oil components [37]. The chemical constituents of M. tomentosa using GC-MS is first reported in this study.

CONCLUSION

The extract of MT augments the antimalarial activity of the chloroquine and to a lesser extent amodiaquine. The therapeutic properties of the extract combination followed a general ameliorative effect in tested animals, hence, an extension of their mean survival rate. This result therefore, present a scientific evaluation for the traditional use of these extracts in combination for malaria treatment. Therefore, further studies are necessary to confirm the toxicological evaluations and pharmacokinetics of Markhamia tomentosa extract in combination with the standard drugs studied.

Compliance with Ethical Standard

Authors contribution statement

Bankole Abimbola Esther: Contributed to all the laboratory work related to antimalarial experiment, collection of plant materials, extraction processes, writing the final Manuscript, contributed to chromatographic and quantification analysis of the plant.

Abiodun Oyindamola: Was involved in the provision of reagents for the antimalarial studies, supervision of laboratory work, preparation of statistical analysis and helping in the interpretation of the results.

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Competing interest statement

The authors declare that they have no competing interest
The animals used in this study were preserved under strict laboratory rules, as approved by the Experimentation Ethics Committee of the College of Medicine, University of Lagos (CM/COM/08/VOL.XXVI) which supported our project.

Table 1: Percentage average parasitemia of response to treatment in the interaction between *Markhamia tomentosa* (MT) and amodiaquine (AQ), artesunate/amodiaquine (AS/AQ) and chloroquine (CQ)

| Treatment                  | Mean Percentage Parasitaemia ± Standard error of Mean |
|----------------------------|------------------------------------------------------|
|                            | D4         | D7         | D12        | D14        | D21        |
| Untreated                  | 8.28±0.660 | 13.13±1.08 | 14.45±0.96 | 16.91±0.79 | -          |
| AQ+ MT 100mg/kg           | 0.51±0.31  | 0.00±0.00  | 0.29±0.18  | 1.98±0.52  | 2.61±0.44  |
| AQ+ MT 250mg/kg           | 1.25±0.55  | 0.00±0.00  | 0.72±0.44  | 3.84±0.80  | 15.88±3.10 |
| AQ+ MT 500mg/kg           | 1.29±0.57  | 0.00±0.00  | 0.00±0.00  | 2.22±0.32  | 6.34±0.80  |
| AQ                        | 0.00±0.00  | 0.00±0.00  | 0.00±0.00  | 5.43±0.65  | 4.37±0.76  |
| AS/AQ + MT 100mg/kg       | 0.54±0.38  | 0.00±0.00  | 0.80±0.49  | 3.35±0.54  |            |
| AS/AQ + MT 250mg/kg       | 2.14±0.74  | 4.61±0.25  | 8.70±1.27  | 14.27±2.06 |            |
| AS/AQ + MT 500mg/kg       | 0.39±0.25  | 0.00±0.00  | 4.61±0.25  | 2.39±0.62  | 15.65±2.67 |
| AS/AQ                     | 0.00±0.00  | 0.00±0.00  | 0.00±0.00  | 3.8±0.70   | 17.17±0.00 |
| CQ + MT 100mg/kg          | 1.37±0.59  | 7.13±1.12  | 12.89±0.12 | 14.77±0.21 |            |
| CQ + MT 250mg/kg          | 3.06±0.81  | 5.62±0.85  | 1.45±0.43  | 9.91±1.26  |            |
| CQ + MT 500mg/kg          | 3.68±0.43  | 0.00±0.00  | 1.12±0.46  | 5.15±0.95  | 11.19±1.22 |
| CQ                        | 0.00±0.00  | 4.93±1.14  | 10.62±0.73 | 10.74±1.50 |            |
| MT 100mg/kg               | 6.45±0.87  | 9.87±1.19  | 24.97±0.04 |            |            |
| MT 250mg/kg               | 4.82±0.71  | 9.28±0.79  | 18.68±3.52 | 10.51±0.00 |            |
| MT 500mg/kg               | 3.67±0.26  | 10.30±1.04 | 9.62±0.69  | 12.90±0.04 |            |

Table 2: Percentage chemosuppression of response to treatment in the interaction between *Markhamia tomentosa* (MT) and amodiaquine (AQ), artesunate/amodiaquine (AS/AQ) and chloroquine (CQ)

| Treatment                  | Mean Percentage Chemosuppression ± Standard Error of Mean |
|----------------------------|----------------------------------------------------------|
|                            | D4 (*TaSEM)         | D7 (*TaSEM)         | D12 (*TaSEM)         | D14 (*TaSEM)         |
| Untreated control          | 0.00                 | 0.00                 | 0.00                 | 0.00                 |
| AQ + 100 mg/kg MT          | 93.82±3.78           | 100.0±0.00           | 97.79±1.28           | 86.8±3.09**          |
| AQ + 250 mg/kg MT          | 82.37±6.73           | 100.0±0.00           | 94.44±3.09           | 77.97±4.74**         |
| AQ + 500 mg/kg MT          | 84.4±6.95            | 100.0±0.00           | 97.18±1.01           | 82.6±1.94**          |
| AQ                        | 100.0±0.00           | 100.0±0.00           | 100.0±0.00           | 66.93±3.99           |
| AS/AQ + 100 mg/kg MT       | 84.67±5.67           | 100.0±0.00           | 100.0±0.00           | 80.92±3.09           |
| AS/AQ + 250 mg/kg MT       | 84.74±5.98           | 100.0±0.00           | 100.0±0.00           | 81.76±3.25           |
| AS/AQ + 500 mg/kg MT       | 84.4±6.89            | 100.0±0.00           | 100.0±0.00           | 86.6±3.66            |
| AS/AQ                      | 100.0±0.00           | 100.0±0.00           | 100.0±0.00           | 82.47±4.03           |
| CQ + 100 mg/kg MT          | 72.35±9.47           | 45.7±8.57            | 0.0                  | 0.0                  |
| CQ + 250 mg/kg MT          | 60.51±9.89*          | 57.25±6.50           | 90.33±3.02*          | 76.6±7.49*           |
| CQ + 500 mg/kg MT          | 55.4±5.24*           | 100.0±0.00           | 91.6±3.19*           | 71.09±5.61*          |
| CQ                        | 100.0±0.00           | 84.4±7.11            | 19.06±6.08           | 0.0                  |
| 100 mg/kg MT               | 18.0±10.63           | 19.27±9.89           | -72.6±0.27           | 0.0                  |
| 250 mg/kg MT               | 41.79±8.59           | 29.40±6.43           | 29.23±24.38          | 0.0                  |
| 500 mg/kg MT               | 53.15±3.18           | 38.24±9.94           | 31.47±9.76           | 23.68±0.26           |

N=5, *P<0.05 = D4 CQ vs CQ+100, 250 or 500 mg/kg, **P<0.05 = D12 & D14 CQ vs CQ+250, CQ+500 mg/kg, *AQ vs AQ+100, 250 or 500 mg/kg
The Journal of Phytopharmacology

Table 3: Average survival rate of treated animals with selected doses of *Markhamia tomentosa* (MT) with different standard drugs

| Treatment                      | Day survival (%) |    |    |    |
|--------------------------------|------------------|----|----|----|
|                               | 7                | 14 | 21 |
| Untreated control              | 100              | 40 | 0  |
| AQ + 100 mg/kg MT              | 100**            |    |    |    |
| AQ + 250 mg/kg MT              | 80**             |    |    |    |
| AQ + 500 mg/kg MT              | 100**            |    |    |    |
| AQ                             | 100**            |    |    |    |
| ASAQ + 100 mg/kg MT            | 100**            |    |    |    |
| ASAQ + 250 mg/kg MT            | 80**             |    |    |    |
| ASAQ + 500 mg/kg MT            | 100**            |    |    |    |
| ASAQ                           | 100**            |    |    |    |
| CQ + 100 mg/kg MT              | 60*              | 20 | 0  |
| CQ + 250 mg/kg MT              | 80*              |    |    |    |
| CQ + 500 mg/kg MT              | 80*              |    | 40*|    |
| CQ                             | 60*              |    |    |    |
| MT 100 mg/kg                   | 80               |    |    |    |
| MT 250 mg/kg                   | 60*              |    |    |    |
| MT 500 mg/kg                   | 80*              |    |    |    |

N=6, *P<0.05 = Untreated control vs treatment groups, †P<0.05 = D14 or 21 untreated control vs D14 or 21 AQ, AQ+MT, ASAQ, ASAQ+MT, CQ+MT, CQ or MT; ‡P<0.05 = CQ vs CQ+500 mg/kg on D21

Table 4: Chemical composition of the oil of *Markhamia tomentosa* leaves

| No. | Compound                        | RT   | KI   | % Composition |
|-----|---------------------------------|------|------|---------------|
| 1   | Naphthalene, 2,6 dimethyl       | 12.20| 1351 | 9.34          |
| 2   | Naphthalene, 1,3 dimethyl       | 12.20| 1351 | 4.67          |
| 3   | Naphthalene, 1,6,7 trimethyl    | 15.00| 1420 | 7.90          |
| 4   | Naphthalene, 1,4,6 trimethyl    | 15.11| 1425 | 4.61          |
| 5   | Naphthalene, 2,3,6 trimethyl    | 15.54| 1443 | 7.74          |
| 6   | Hexadecane                      | 17.14| 1506 | 35.55         |
| 7   | Pentadecane, 7-methyl           | 17.14| 1534 | 5.47          |
| 8   | 10—methyl nonadecane           | 17.14| 1534 | 5.47          |
| 9   | Tridecane, 5- propyl            | 19.72| 1649 | 3.14          |
| 10  | Octadecane                      | 21.92| 1881 | 10.9          |
| 11  | Heptadecane, 8- methyl          | 21.92| 1881 | 5.44          |
| 12  | Phenanthrene,4-methyl           | 23.59| 1788 | 0.93          |
| 13  | 1H- cyclopropa(l) Phenanthrene, 1a, 9b- dihydro | 23.59| 1788 | 0.93          |
| 14  | Anthracene, 1- methyl           | 23.69| 1793 | 0.98          |
| 15  | Heptadecane                     | 24.15| 2024 | 20.49         |

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