Abstract: A method for preparing an immunogen to trigger production of anti(9-methoxy corynantheidine) antibody for a use in detecting presence of mitragynine in humans.
before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
A METHOD FOR PREPARING AN IMMUNOGEN AND A USE THEREOF

FIELD OF INVENTION
The present invention relates to a method for preparing an immunogen to trigger production of anti(9-methoxy-corynantheidine) antibody and a use thereof for detecting presence of mitragynine in humans.

BACKGROUND OF INVENTION
Mitragyna speciosa is a herbal plant which indigenous to Southeast Asian countries notably in Thailand, Malaysia, and Myanmar. This plant is known as 'Kratom' in Thailand; 'Biak-biak' and 'Ketum' in Malaysia, where it is used by local people as traditional medicine to cure illnesses which includes fever, cough, diarrhea, hypertension, and counter fatigue. Besides, it can act as a substituent for opium. It is also known to prevent withdrawal symptoms in an opiate dependent.

Mitragynine, or 9-methoxy-corynantheidine, is an alkaloid which accounts for over 66% from total alkaloids extract of Mitragyna speciosa. In small doses, mitragynine exhibits stimulant effects whereas it shows opiate-like effects in higher doses. Some pharmacological studies have revealed that mitragynine present plenty of properties which include anti-inflammatory, antioxidant, antimicrobial, antidiarrheal, antidepressant, antinociceptive, antitussive, anaesthetic, stimulant, analgesic, and also inhibit ethanol withdrawal symptoms.

Although Mitragyna speciosa has exhibit properties in prevention of opiate addiction and withdrawal symptoms, it has been proven to be addictive itself. In other words, use of Mitragyna speciosa can also indicate abuse potential. In recent years, there has been a growing trend among drug addicts who use ketum leaves to get high when they are unable to get their regular supply of cannabis or heroin, thus, driven numerous ways to overcome this issue. A method for treating withdrawal from addictive compounds was disclosed in US Patent Application No. US 2010/00209542.
In efforts to combat addiction to mitragynine, various methods of detecting presence of alkaloids have been developed in the past. In US Patent Application No. US 2005/0182257, method of detecting and extracting alkaloids using reversed phase separation chromatography is described herein. Although chromatographic methods like GC-MS and HPLC-UV are able to detect and confirm present of drugs quantitatively, however, application of this method is restricted by high cost, personnel trained is required, only a single sample is determined in a time, and sample preparation is required before analysis is carried out.

Subsequently, detection kit for mitragynine in urine sample based on an Enzyme-Linked Immunosorbent Assay (ELISA) principle is developed. ELISA is a preferred method to perform quantitative analysis of mitragynine as it is economical and sensitively reliable. Furthermore, ELISA method may not suffer deleterious effects from the presence of other components of the sample (matrix effects) due to interfering substances including pigments from biological samples are washed from the plate before colour development, hence making this method having lower risk of interference than a homogeneous assay.

However, this method is hampered by the unavailability of antibody-based immunoassay tests. To develop immunoassay based tests, specific antibodies, either monoclonal or polyclonal need to be raised.

Accordingly, a need still exists for a method for preparing an immunogen to trigger production of anti(9-methoxy-corynantheidine) antibody for a use in detecting presence of mitragynine in humans.
SUMMARY OF INVENTION

Accordingly, the present invention relates to a method for preparing an immunogen to trigger production of anti(9-methoxy-corynantheidine) antibody for a use in detecting presence of mitragynine in humans wherein the method includes steps of i) preparing a mixture solution of 1.1 part of p-aminobenzoic acid (PABA) in hydrochloric acid solution and 1 part of sodium nitrite in distilled water solution; ii) stirring the mixture solution; iii) preparing a mixture by adding the above prepared mixture solution into a 1 part of mitragynine in mixture of acetic acid and distilled water solution to allow formation of hapten PABA-mitragynine; iv) stirring the mixture; v) adding distilled water into the mixture; vi) conducting extraction onto the mixture with an organic solvent; vii) separating the organic layer containing hapten PABA-mitragynine from the mixture; viii) absorbing moiety of the organic layer; ix) removing excess organic solvent; x) conducting purification to produce purified hapten PABA-mitragynine; xi) adding 1.4 part of purified hapten PABA-mitragynine into a charge mixture of 1 part of N-Hydroxy Succinimide (NHS) and 1 part of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in dimethylformamide (DMF); xii) stirring the mixture obtained in step xi); xiii) adding 50 parts of the mixture into 1 part of bovine serum albumin (BSA) in sodium bicarbonate solution to form PABA-MG-BSA solution; xiv) stirring the PABA-MG-BSA solution; and xv) conducting dialysis to leave purified PABA-MG-BSA.

The present invention also provides a method for preparing an immunogen to trigger production of anti(9-methoxy-corynantheidine) antibody for a use in detecting presence of mitragynine in humans wherein the method includes steps of i) dissolving cationic bovine serum albumin (CBSA) in 2 parts of distilled water; ii) adding 1 part of sodium acetate solution and 2 parts formaldehyde solution into step (i); iii) preparing a mitragynine solution which was dissolved in 3 parts of dimethyl sulfoxide (DMSO) and 1 part of acetic acid; iv) adding the solution obtained in step (iii) into a CBSA-formaldehyde solution; v) stirring the solution obtained in step (iv); vi) adding 6 parts of DMSO-acetic acid mixture to form hapten Mannich-Mitragynine-CBSA; vii) stirring the hapten
Mannich-Mitragynine-CBSA mixture; and viii) conducting dialysis to leave purified Mannich-Mitragynine-CBSA.

**BRIEF DESCRIPTION OF DRAWINGS**

The drawings constitute part of this specification and include an exemplary or preferred embodiment of the invention, which may be embodied in various forms. It should be understood, however, the disclosed preferred embodiments are merely exemplary of the invention. Therefore, the figures disclosed herein are not to be interpreted as limiting, but merely as the basis for the claim and for teaching one skilled in the art of the invention.

In the appended drawings:

FIG. 1 illustrates a graph of specific antibody binding / maximum antibody binding (B/B₀%) vs. Concentration of Mitragynine in ng/ml

**DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

Detailed descriptions of preferred embodiments of the present invention are disclosed herein. It should be understood, however, that the embodiments are merely exemplary of the present invention, which may be embodied in various forms. Therefore, the details disclosed herein are not to be interpreted as limiting, but merely as the basis for the claim and for teaching one skilled in the art of the invention.

In the present invention, the first step of producing immunogen to trigger production of anti(9-methoxy-corynantheidine) antibody against mitragynine is synthesis of mitragynine-p-aminobenzoic acid (PABA-Mitragynine).

Firstly, a p-aminobenzoic acid (PABA) solution is prepared with PABA in hydrochloric acid (HCl), preferably the HCl in a volume ranging from 0.5ml to 1.5ml, while separately a sodium nitrite solution is prepared with sodium nitrite (NaNC^) in distilled water, preferably the distilled water in a volume ranging...
from 0.2ml to 1ml. Both prepared solutions are cooled to 4°C and stirred separately for at least 30 minutes.

Next, 1 part of sodium nitrite solution is added into 1.1 part of PABA solution at a temperature ranging from 3-5°C, preferably at 4°C, to form a mixture solution and is stirred for at least 45 to 75 minutes, preferably at 60 minutes. To assure formation of diazonium salt, napthol is added into 60µl of mixture solution. If diazonium salt is formed, the mixture solution will turn red in color.

In another embodiment, hapten preparation involves a method of modifying the mitragynine molecule to produce suitable functional groups such as -COOH, NH₂, SH, OH, followed by coupling the modified structure to a carrier protein. The method described herein is by adding para-aminobenzoic acid (PABA) in order to introduce a carboxylic acid group into mitragynine which having aromatic ring.

Typically, a mitragynine solution is prepared with mitragynine in glacial acetic acid (CH₃COOH), preferably the CH₃COOH in a volume ranging from 1ml to 2ml, and distilled water, preferably the distilled water in a volume ranging from 0.5ml to 1.5ml. The mitragynine solution is then cooled to 4°C and stirred for at least 45 to 75 minutes, preferably at 60 minutes.

Next, 1 part of the mixture solution prepared earlier is added into 1 part of the mitragynine solution to allow formation of hapten PABA-mitragynine. The mixture is stirred for at least 2 to 4 days, preferably at 4 days, at a temperature ranging from 3-5°C, preferably at 4°C.

Then, 20ml of distilled water is added into the mixture. Extraction is carried out using an organic solvent, preferably but not limiting to, methylene chloride (CH₂Cl₂). After that, the extraction is repeated once again. Next, the organic layer is separated after the extraction and collected. Sodium sulphate (Na₂SO₄) is added to the organic layer to absorb moiety. Excess solvent is then removed by evaporation using rotary evaporator.
Thin layer chromatography (TLC) is employed by using solvent chloroform: methanol at a ratio of (9:1) for verification. Subsequently, purification step is carried out by using silica gel column chromatography. A pure hapten PABA-Mitragynine produced is confirmed by LC-MS/MS.

The modified molecule is then conjugated to a carrier protein like bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), thyroglobulin or other large biological molecules to produce a hapten.

Typically, a charge mixture of 1.4 part of hapten PABA-Mitragynine, 1 part of N-Hydroxy Succinimide (NHS) and 1 part of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in dimethylformamide (DMF), preferably the DMF a volume ranging from 0.2ml to 0.3ml is prepared. The mixture is stirred for at least 23 to 25 hours, preferably for 24 hours at a temperature ranging from 23-27°C, preferably at 25°C.

Then, PABA-MG-BSA solution is prepared by adding 50 parts of the mixture into 1 part of the solution of bovine serum albumin (BSA) in 2ml sodium bicarbonate and is stirred for at least 4-6 hours, preferably at 5 hours at a temperature ranging from 23-27°C, preferably at 25°C. The PABA-MG-BSA solution is then purified by conducting dialysis using 0.01 M Phosphate Buffered Saline (PBS).

Purified PABA-MG-BSA is used as an immunogen trigger production of anti(9-methoxy-corynantheidine) antibody against mitragynine in mammals.

In another embodiment, hapten mitragynine can also be prepared by condensation of active hydrogen present in mitragynine with an amine-containing compound present in carrier protein in the presence of formaldehyde to form stable crosslinks. 13mg of cationic BSA are dissolved in 2 parts of distilled water. Then, 1 part of 3M sodium acetate solution and 2 parts of 8% formaldehyde solution are added.
Next, 5mg of mitragynine dissolved in 3 parts of dimethyl sulfoxide (DMSO) and 1 part of acetic acid. This solution was added to the above CBSA-formaldehyde solution with constant stirring. After that, 6 parts of 1:1 DMSO-acetic acid mixture was then added to form hapten Mannich-Mitragynine-CBSA. The mixture was stirred for at least 23 to 25 hours, preferably for 24 hours at a temperature ranging from 23-27 °C, preferably at 25 °C.

The next day, the solution was dialyzed for two days against 0.01 M phosphate buffer (PBS). The synthesized mitragynine hapten was then kept in freezer around -20 °C before use.

Suitable mammals such as rabbits, sheep, or goats are used as host for eliciting polyclonal antibodies. For monoclonal antibody production, a Balb C mouse is used. In the present invention, rabbits and mice are immunized with the immunogen PABA-MG-BSA solution or Mannich-Mitragynine-CBSA. The immunogen is added with complete freund adjuvant for first immunization and incomplete freund adjuvant for the following boosting.

A dose of purified immunogen PABA-MG-BSA with complete freund adjuvant is injected into a host mammal as first immunization. Next, a dose of purified immunogen PABA-MG-BSA with incomplete freund adjuvant is injected into the host mammal as first booster on 28th day after the first immunization. On 7th day after the first booster is carried out, a blood sample is collected from the host animal. The blood sample is then centrifuged to separate serum containing anti(9-methoxy-corynantheidine) antibody from the blood. Saturated ammonium sulfate (SAS) is then added into the serum to purify antibody obtained.

Specificity of antibody obtained is determined using checker board dilution.

A second booster is carried out on 42nd day after the first booster.

The following examples are included to demonstrate preferred embodiments of the invention.
**EXAMPLES:**

**Preparation of hapten mitragynine**

A. *Synthesis of Hapten (PABA)-Mitragynine*

PABA solution was prepared with 13.7mg p-aminobenzoic acid (PABA) in 1ml of 2N hydrochloric acid (HCl), while sodium nitrite solution was prepared with 6.2mg sodium nitrite (NaNO₂) in 200μl of distilled water. Both prepared solutions were cooled to 4°C and stirred separately for at least 30 minutes.

Then, 200μl sodium nitrite solution was added into PABA solution to form a mixture solution at 4°C and the mixture solution was stirred for at least one hour. To assure formation of diazonium salt, napthol was added into 60μl of mixture solution. A colour red was observed on the solution indicated formation of diazonium salt.

Separately, a mitragynine solution was prepared with 36mg mitragynine in 1.5ml glacial acetic acid (CH₃COOH) and 1ml distilled water. The mitragynine solution was then cooled to 4°C and stirred for at least 30 minutes.

Next, the mixture solution prepared earlier was added into the mitragynine solution to form a mixture. The mixture was stirred for four days at 4°C.

After four days, 20ml of distilled water was added into the mixture. Extraction was carried out using an organic solvent, preferably but not limiting to, methylene chloride (CH₂Cl₂). After that, the extraction was repeated once again. Next, the organic layer was separated after the extraction and collected. Sodium sulphate (Na₂SO₄) was added to the organic layer to absorb moiety. Excess solvent was then removed by evaporation using rotary evaporator.

Thin layer chromatography (TLC) was employed by using solvent chloroform: methanol at a ratio of (9:1) for verification. Subsequently, purification step was carried out by using silica gel column chromatography. A pure hapten PABA-Mitragynine produced was confirmed by LC-MS/MS.
A charge mixture of 3.9 mg hapten of Mitragynine, 0.6 mg N-Hydroxy Succinimide (NHS) and 0.8 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 240 µl dimethylformamide (DMF) was prepared. The mixture was stirred for 24 hours at 25°C. Then, 120 µl of mixture was added into a solution of 5 mg bovine serum albumin (BSA) in 0.13 M 2 ml sodium bicarbonate to form PABA-MG-BSA solution and was stirred for 5 hours at 25°C. The solution was then purified using dialysis with 0.01 M Phosphate Buffered Saline (PBS).

B. Synthesis of Hapten Mannich-Mitragynine-CBSA

13 mg of cationic BSA were dissolved in 250 µl of distilled water. Then, 125 µl of 3 M sodium acetate solution and 250 µl of 8% formaldehyde solution were added. Next, 5 mg of mitragynine were dissolved in 375 µl of dimethyl sulfoxide (DMSO) and 125 µl of acetic acid. This solution was added to the above CBSA-formaldehyde solution with constant stirring. After that, 750 µl of 1:1 DMSO-acetic acid mixture was then added to form Mannich-Mitragynine-CBSA. The mixture was stirred for at least 23 to 25 hours, preferably for 24 hours at a temperature ranging from 23-27°C, preferably at 25°C.

The next day, the solution was dialyzed for two days against 0.01 M phosphate buffer (PBS). The synthesized mitragynine hapten was then kept in freezer around -20°C before use.

Immunization

The prepared PABA-MG-BSA or Mannich-Mitragynine-CBSA was used as an immunogen. Rabbits and mice were immunized with the prepared immunogen PABA-MG-BSA or Mannich-Mitragynine-CBSA. The immunogen was added with complete freund adjuvant for first immunization and incomplete freund adjuvant for the following boosting.
For rabbits, a dosage of 1mg/ml immunogen was used. The rabbits were injected after four weeks of first immunization. Subsequently, following boosting was conducted every six weeks and collection of blood was done after seven days of each boosting. Blood was collected and allowed to clot for at least 45-75 minutes, preferably at 60 minutes, at a temperature ranging from 22-28°C, preferably at 25°C. Then, the blood was centrifuged at 10,000rpm for at least 5-15 minutes, preferably at 10 minutes to separate serum from the blood. After that, 250µl solution of anti(9-methoxy-corynantheidine) antibody was prepared by adding saturated ammonium sulfate (SAS) onto the serum. Specificity of antibody obtained is determined using checker board dilution.

A second booster is conducted on the 42nd day after the first booster. For mice, a dosage of 50-100µg immunogen is used.

The anti(9-methoxy-corynantheidine) antibody produced can be used in a diagnostic kit for detecting presence of mitragynine in human body fluid samples of urine, blood or saliva.

ELISA kit and test strip for detection of mitragynine is developed and validated by using LC-MS/MS. First stage is to synthesis a mitragynine hapten in order to generate antibody against mitragynine which can be used for developing immunoassay. Firstly, polyclonal antibody is raised. For detection of mitragynine, ELISA principle is employed. A microtitre plate is coated with anti-mitragynine antibody to capture the antigen (mitragynine) when present. Typically, sample and antigen labelled with horse radish peroxidase (HRP) as enzyme is added to compete as an analyte for antibody binding site. Then, a colourless second substrate is added which initiates a reaction is converted into a colour product (blue) by the HRP in inversely proportion to the amount of analyte bound. The reaction is stopped upon addition of acidic solution which turns the solution from blue to yellow. Optical density (O.D.) of the yellow color is read at A450 on a microtiter plate reader. The higher the sample antigen concentration is present, the weaker the strength of the yellow colour. On the other hand, monoclonal antibodies obtained from mice are used to develop lateral flow immunoassay against mitragynine. A well characterized hapten-protein conjugates
Mitragynine-BSA as receptor in an immunochromatographic dipstick will be synthesized. Lastly, quantitative analysis of mitragynine detection using LC-MS/MS is performed and is used to validate the immunoassay.

5 Results and Discussion:

Specific antibodies against mitragynine are obtained. Results showed good sensitivity and specificity against mitragynine as shown in TABLE 1, using a direct immunoassay format with mitragynine-PABA-HP synthesized using the method described above.

<table>
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<th>[Mitragynine] in ng/ml</th>
<th>Absorbance</th>
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<tr>
<td>0</td>
<td>1.376</td>
<td>100</td>
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<tr>
<td>1</td>
<td>1.273</td>
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<tr>
<td>50</td>
<td>0.729</td>
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<tr>
<td>100</td>
<td>0.587</td>
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</tr>
<tr>
<td>500</td>
<td>0.389</td>
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</tr>
<tr>
<td>5000</td>
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<td>10.2</td>
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While embodiments and examples of the present invention have been illustrated and described, it is not intended that these embodiments and examples illustrate and describe all possible forms of the present invention. Rather, words used in the specification are words of description rather than limitation and various changes may be made without departing from the scope of the invention.
CLAIMS

1. A method for preparing an immunogen to trigger production of anti (9-methoxy-corynantheidine) antibody for a use in detecting presence of mitragynine in humans wherein the method includes steps of:

i) preparing a mixture solution of 1.1 part of p-aminobenzoic acid (PABA) in hydrochloric acid solution and 1 part of sodium nitrite in distilled water solution;

ii) stirring the mixture solution;

iii) preparing a mixture by adding the above prepared mixture solution into a 1 part of mitragynine in mixture of acetic acid and distilled water solution to allow formation of hapten PABA-mitragynine;

iv) stirring the mixture;

v) adding distilled water into the mixture;

vi) conducting extraction onto the mixture with an organic solvent;

vii) separating the organic layer containing hapten PABA-mitragynine from the mixture;

viii) absorbing moiety of the organic layer;

ix) removing excess organic solvent;

x) conducting purification to produce purified hapten PABA-mitragynine;

xi) adding 1.4 part of purified hapten PABA-mitragynine into a charge mixture of 1 part of N-Hydroxy Succinimide (NHS) and 1 part of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in dimethylformamide (DMF);

xii) stirring the mixture obtained in step xi);

xiii) adding 50 parts of the mixture into 1 part of bovine serum albumin (BSA) in sodium bicarbonate solution to form PABA-MG-BSA solution;

xiv) stirring the PABA-MG-BSA solution; and

xv) conducting dialysis to leave purified PABA-MG-BSA.
2. A method as claimed in Claim 1 wherein the immunogen is PABA-MG-BSA.

3. A method as claimed in Claim 1 wherein step (ii) is conducted at a time ranging from 45 to 75 minutes.

4. A method as claimed in Claim 1 wherein step (iv) is conducted for a period ranging from 2 to 4 days and at a temperature ranging from 3 to 5°C.

5. A method as claimed in Claim 1 wherein the distilled water in step v) is added at 20ml.

6. A method as claimed in Claim 1 wherein an organic solvent in step vi) is methylene chloride.

7. A method as claimed in Claim 1 wherein step viii) is carried out by adding sodium sulphate.

8. A method as claimed in Claim 13 wherein step ix) is carried out using evaporation.

9. A method as claimed in Claim 13 wherein step x) is carried out using silica gel column chromatography.

10. A method as claimed in Claim 1 wherein step (xii) is conducted for a period ranging from 23 to 25 hours and at a temperature ranging from 23 to 27°C.

11. A method as claimed in Claim 1 wherein step (xiv) is conducted for a period ranging from 4 to 6 hours and at a temperature ranging from 23 to 27°C.
12. A method for preparing an immunogen to trigger production of anti(9-methoxy-corynantheidine) antibody for a use in detecting presence of mitragynine in humans wherein the method includes steps of:

i) dissolving cationic bovine serum albumin in 2 parts of distilled water;

ii) adding 1 part of sodium acetate solution and 2 parts formaldehyde solution;

iii) adding a mitragynine solution which was redissolved in 3 parts of dimethyl sulfoxide (DMSO) and 1 part of acetic acid;

iv) adding the solution obtained in step (iii) into a CBSA-formaldehyde solution;

v) stirring the solution obtained in step (iv);

vi) adding 6 parts of DMSO-acetic acid mixture to form hapten Mannich-Mitragynine-CBSA;

vii) stirring the hapten Mannich-Mitragynine-CBSA mixture;

viii) conducting dialysis to leave purified Mannich-Mitragynine-CBSA.

13. A method as claimed in Claim 12 wherein the immunogen is Mannich-Mitragynine-CBSA.

14. A method as claimed in Claim 12 wherein step v) is conducted for a period of at least 23 to 25 hours, and at a temperature ranging from 23 to 27°C.

15. A method as claimed in Claim 12 wherein the DMSO-acetic acid mixture in step vi) is in a ratio of 1:1.

16. A method as claimed in Claim 12 wherein step (vii) is conducted for a period of at least 23 to 25 hours, and at a temperature ranging from 23 to 21°C.
17. A method as claimed in Claim 12 wherein step (viii) is conducted using 0.01 M phosphate buffer (PBS).

18. A use of an immunogen to trigger production of anti(9-methoxy-corynantheidine) antibody as claimed in Claims 1 to 17 in a diagnostic kit for detecting presence of mitragynine in human body fluid samples.
FIG. 1

\[ y = -10.08 \ln(x) + 91.798 \]

\[ R^2 = 0.9828 \]
INTERNATIONAL SEARCH REPORT

International application No.
PCT/MY2013/000052

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/113 (2006.01)  C07D 455/00 (2006.01)  G01N 33/577 (2006.01)  G01N 33/94 (2006.01)  G01N 33/53 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

STN: Registry - Registry Number ,098-40-2 and STN: MEDLINE, WPIDS, HCAPLUS, BIOSIS and BIOTECHABS - keywords based on mitragyna speciosa, kratom, kratum, kratom or indole alkaloid and antibody, immunogen, antisera, hapten, antigen or immunoassay.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
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Further documents are listed in the continuation of Box C

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<th>Special categories of cited documents:</th>
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<td>&quot;A&quot; document defining the general state of the art which is not considered to be of particular relevance</td>
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<td>&quot;E&quot; earlier application or patent but published on or after the international filing date</td>
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<td>&quot;L&quot; document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td>
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<td>&quot;P&quot; document published prior to the international filing date but later than the priority date claimed</td>
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Date of the actual completion of the international search: 22 July 2013
Date of mailing of the international search report: 22 July 2013

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<table>
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