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Pretreatment of Hocmon Betel Leaf to Produce Essential Oils with High Phenolic Content

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Chavibetol **1** instead of eugenol or isoeugenol, chavibetol acetate **2** instead of eugenol acetate and 4-allylpyrocatechol diacetate **3** were unequivocally identified by us as the main phenolic compounds in Hocmon betel leaf essential oil (*Piper betle* L.). It was also found that oil yield and its phenolic content varied with the time of collection and the mode of leaf pretreatment. In 2011, at the 14th Asian Chemical Congress in Bangkok, we reported for the first time the transformation of 4-allylpyrocatechol diacetate **3** into chavibetol **1** resulting in a substantial decrease in oil yield and in the total phenolic content by comparison of the products which were respectively obtained by hydrodistillation in unsalted water and in NaCl saturated solution. In this communication, further experiments were performed in more details to show that this transformation would be of enzymatic origin and occurred only in the presence of betel leaf via the intermediate undistilled water soluble 4-allylpyrocatechol **4**. The role of NaCl was to block effectively the enzymatic reactions, thus 4-allylpyrocatechol diacetate **3** largely remained unchanged**.** In unsalted water, these reactions readily occurred and concurrently produced a substantial increase of **1** and a drastic decrease of **3** while large amount of undistilled **4** led to a decrease of betel oil yield and of its phenolic content. As it was known that the antimicrobial activity of betel essential oil increased with its phenolic content, Statgraphics Centurion XVI.II was used to define the experimental conditions to provide better yield of oil which must also contain a greater percentage of the phenolic compounds and particularly the more stable 4-allylpyrocatechol diacetate **3** with respect to oxidation in view of its possible use in cosmetic products.

Key words: Betel leaf oil; yield; phenolic components; transformation; chavibetol acetate; 4-allylpyrocatechol diacetate; chavibetol; maximum yield

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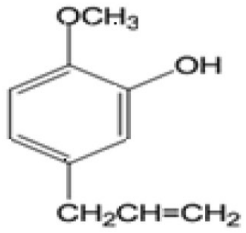
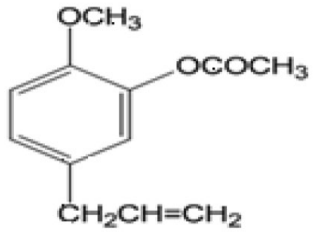
In a previous communication [1], we unequivocally identified the phenolic components in Hocmon betel leaf essential oil as chavibetol **1**, chavibetol acetate **2** and 4-allylpyrocatechol diacetate **3** (APC diacetate). In 2011, at the 14th Asian Chemical Congress in Bangkok [2], we reported for the first time the drastic variation of oil yield and its phenolic content when hydrodistillation was performed in saturated NaCl water or in unsalted water (Table 1). By comparison with case 3, results showed in case 1 a large decrease of oil content, a drastic decrease of **3,** a smaller diminution of **2** and a quite significant increase of **1**. In case 2, a decrease of **3** and an increase of **1** were also observed but to a lesser extent.

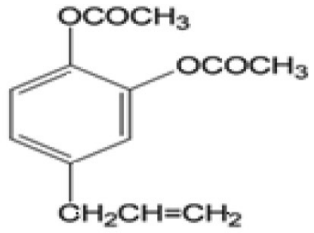
We tentatively explained the increase of **1** by an enzymatic hydrolysis of **3** into an intermediate water soluble polar compound,



Chavibetol **1**



Chavibetol acetate **2**



4-allylpyrocatechol diacetate **3**

Table 1. Oil yields and variation of the percentages of phenolic compounds in betel oil [1].

Experiment	Leaves collected on 5 January, 2011	Oil, mg (% of dried leaves at 105°C)	**1** (% area GC-MS)	**2** (% area GC-MS)	**3** (% area GC-MS)
1	Leaves (300 g) ground in 900 ml of distilled water for 1 h before hydrodistillation for 3 h	282 (0.656)	26.19	13.06	3.18
2	Leaves (300 g) ground in 900 ml of 10 % NaCl solution for 1 hr before hydrodistillation for 3 h	441 (1.026)	14.42	22.85	25.19
3	Leaves (300 g) ground in 900 ml of saturated NaCl solution for 1 h before hydrodistillation for 3 h	875 (2.035)	5.66	21.98	60.55

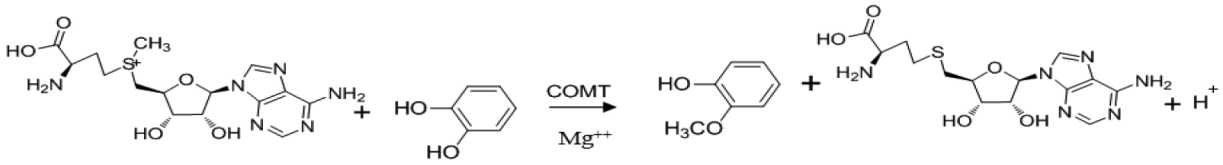
4-allylpyrocatechol **4** (APC), which could not be hydrodistilled; compound **4** was then partially methylated selectively at OH in para position with respect to the allyl substituent. Indeed, **4** was isolated from the water remaining in the flask after hydrodistillation and unequivocally identified by GC-MS, LC-MS and NMR. It was well known that in some plants, the enzyme catechol O-methyl transferase (COMT) existed and in the presence of Mg^{++} catalyzed the transfer of the methyl group of S-adenosyl-L-methionine (AdoMet) present in the plant to one of the OH group of substituted catechols to give the O-methylated derivatives. The reaction could be due to a nucleophilic attack of the phenolic OH on the electron-deficient methyl group of AdoMet, the meta/para ratio depended on the nature of the substituent and the nucleophilicity of the OH group [3–7]. Increase of methylation at the para phenolic OH was observed with relatively non-polar substituent (meta/para ratio: 0.4 with C_6H_5CH=CH- substituent) [3,5]. In our case, para methylation occurred solely and was due to the increased nucleophilicity of the para OH through hyperconjugation.

In this communication, experiments were quantitatively performed to support the hypothesis of enzymatic intervention, then Statgraphics Centurion XVI.II was used to determine the experimental conditions to obtain highest yield of betel oil with very high content of phenolic compounds and particularly of the more stable APC diacetate toward oxidation.

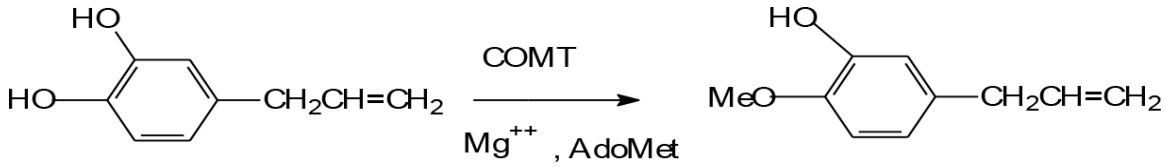
MATERIALS AND METHODS

Hydrodistillation of Betel Leaf Essential Oil

Fresh leaves were treated somewhat differently from the process we described in our previous communications. They were collected from 6 a.m. - 7 a.m. in Hocmon betel garden and brought to the laboratory around 8 a.m. Leaves (400 grams) were ground first at room temperature with 400 ml of unsalted or appropriately salted water for 1 min and added to one 2 litre distilling flask, then 600 ml of hot or boiling and suitably salted water were very quickly added to obtain finally in less than 15 sec unsalted or half-saturated (18% NaCl) or saturated NaCl (36% NaCl) mixtures at chosen temperatures



S-Adenosyl-L-methionine Catechol Guaiacol S-Adenosyl-L-homocysteine



APC **4** Chavibetol **1**

30°C, 50°C, 70°C. The flask was then heated at a suitable rate so that in every case, hydrodistillation started after 20 min. Hydrodistillation lasted for 3 h when performed in saturated NaCl solution. For the other cases, it was performed for 2 h, then the mixture in the distilling flask was saturated with salt and distillation continued for one more hour to ensure complete collection of the essential oil. Oil yields were calculated on the basis of fresh leaves. Temperature was chosen in the range 30°C–70°C, because enzyme would normally cease its activity starting around 70°C.

Isolation and Purification of 4-allylpyrocatechol 4 **and Chavibetol** 1

(a) For 4-allylpyrocatechol **4** (APC) isolation, extraction of the water after distillation with ether and clean-up by successive column chromatography on silicagel (fraction hexane : ethyl acetate in the ratio 9 : 1) and C18 sorbent (fraction MeOH : H_2O in the ratio 1 : 1) gave pure white crystalline compound **4** which was identified by full scan GC-EIMS [only 1 peak at 28.016 min, *m/z* = 150 (100%), 133, 131, 123, 103, 77 and and LC - MS/MS - ESI(-) [*m/z* = 148.96 (M-1), 122, 108 (from further loss of $CH{=}CH_2$ and $–CH_2CH{=}CH_2$)], ^{1}H NMR (500 MHz, MeOD) [δ_H ppm; 3 aromatic protons: 6.68 (1H; *d*; *J* = 8 Hz; H-6); 6.62 (1H; *d*; *J* = 2.0 Hz; H-3); 6.49 (1H; *dd*; *J* = 8 Hz; *J* = 2 Hz; H-5); -CH: 5.89 – 5.97 (1H; m; H-β); CH_2: 4.98 – 5.05 (2 H; m; H-γ); CH_2: 3.22 (2 H; d; *J* = 7 Hz; H-α)]

(b) Chavibetol **1** was obtained by treatment of betel oil sample (being rich in **1)** with 1M NaOH at 0°C, extraction with hexane to eliminate the other oil components, then neutralization of the aqueous solution with 10% H_2SO_4, re-extraction with ether, purification by chromatography on silica-gel (elution with hexane : ethyl acetate in the ratio 95: 5). Full scan GC-MS chromatogram showed peak at the retention time 25.3 min – 25.4 min, *m/z* = 164 (100%), (purity: 99.43%). ^{1}H NMR (500 MHz, $CDCl_3$) [δ_H ppm; 3 aromatic protons: 6.78 (1H; *d*; *J* = 8.0 Hz; H-6); 6.79 (1 H; *d*; *J* = 2.0 Hz; H-3); 6.67 (1 H; *dd*; *J* = 8Hz; *J* = 2.0 Hz; H-5); -OCH_3: 3.87 (3 H; s); OH: 5.62; -$CH+_2$: 3.3 (2.0 H; d; *J* = 6.5 Hz; H-α); -CH: 5.91 – 5.98 (1 H; m; H-β)]; -CH_2: 5.04 – 5.10 (2.0 H; m; H-γ)]

Synthesis of Chavibetol Acetate 2 **and 4-allylpyrocatechol Diacetate** 3

(a) Compound **2** was obtained by reacting chavibetol **1** with excess of acetic anhydride in the presence of pyridine. Purification was obtained by column chromatography

on silicagel (elution with hexane : ethyl acetate 97 : 3), GC-MS chromatogram showed one peak at the retention time 29.40 min – 29.50 min, *m/z* = 206, 164 (100%) and *m/z* = 43 characteristic of the CH_3CO group (yield 82%, purity: 99.65%).

(b) Compound **3** was also obtained by reacting 4-allylpyrocatechol with excess of acetic anhydride in the presence of pyridine. Purification was obtained by column chromatography on silicagel (elution with hexane : ethyl acetate 97 : 3). Full scan GC-MS chromatogram showed peak at the retention time 32.10 min – 32.20 min, m/z = 234, 192, 150 (100%), (yield 84%, purity: 98%). ^{1}H NMR (500 MHz, MeOD), [δ_H ppm; 3 aromatic protons: 7.13 (1H; *d*; *J* = 8.2 Hz; H-6); 7.1 (1H; *dd*; *J* = 8.5 Hz; *J* = 2 Hz; H-5); 7.04 (1H; *d*; *J* = 2.0 Hz; H-3); -CH_3: 2.26 (6H; s); -CH_2: 3.39 (2 H; *d*; *J* = 6.5 Hz; H-α); -CH: 5.93 – 6.01 (1H; m; H-β); -CH_2: 5.08 – 5.13 (2 H; m; H-γ)].

Determination of Compounds 1**,** 2**,** 3 **in Betel Oil and** 4 **in Water after Hydrodistillation**

Linear calibration curves representing GC-MS peak intensities versus concentrations were obtained with standard solutions of **1**, **2**, **3, 4** in acetone with isoeugenol as internal standard:

- For chavibetol **1**, y = 0.065x – 0.017; R^2 = 1.000, x: 2.05 ppm – 51.2 ppm
- For chavibetol acetate **2**, y = 0.120x + 0.001; R^2 = 0.999, x: 2.06 ppm – 51.41 ppm
- For 4-allylpyrocatechol diacetate **3**, y = 0.129 + 0.032x; R^2 = 0.998, x: 1.46 ppm – 36.6 ppm; and
- For 4-allylpyrocatechol **4**, y = 0.017x – 0.666; R^2 = 0.995, x: 43.73 ppm – 218.63 ppm.

With these calibration curves, compounds **1**, **2**, **3, 4** were determined by GC-MS. APC remaining in water after hydrodistillation was isolated by supported liquid extraction SLE using Chromabond XTR as adsorbent and analyzed by GC-MS.

QUANTIFICATION RELATED TO THE TRANSFORMATION OF 4-ALLYLPYROCATECHOL DIACETATE **3** AND CHAVIBETOL ACETATE **2** INTO CHAVIBETOL **1**

(a) In each experiment of hydrodistillation, the contents of each compound **1**, **2**, **3** and **4** were precisely calculated by utilizing the precited calibration curves. The increase of **1** was attributed to the hydrolysis of **2** and to the methylation of a part of APC coming from the hydrolysis of **3**. The theoretical quantity of unmethylated APC was calculated and then compared to the experimental value of APC remaining in water in the flask after hydrodistillation.

(b) As Mg^{++} was needed in the enzymatic methylation, its influence on the extent of the transformation of **4** into **1** was also examined [6,7]. Addition of $MgCl_2.6H_2O$ to the mixture of ground betel leaves before hydrodistillation would increase chavibetol content in betel oil.

(c) In another experiment, water after hydrodistillation containing unmethylated APC from a previous batch was used to perform a second hydrodistillation with ground betel leaves. More chavibetol **1** from methylation of pre-existing APC would be expected if enzymatic reaction occurred in the presence of betel leaves. All experiments were conducted at 30°C as starting temperature.

Experimental Conditions to Obtain Highest Yield of Betel Oil with very High Content of Phenolic Components and APC Diacetate

Statgraphics Centurion XVI.II was used for optimization calculations based on the percentages of area relative to compounds **1, 2, 3** on GCMS chromatograms. The aim is to find the experimental conditions to get better yield of betel leaf essential oil containing a greater percentage of antibacterial phenolic compounds and the relatively stable compound **3** toward oxidation in the temperature range 30°C – 70°C and salt content 0% – 36% in view of its possible use in cosmetic production.

RESULTS AND DISCUSSION

All experiments were performed with leaves collected during 2 months July and August 2014. It is worth to note that betel oil yield and its chemical composition depend significantly on the month and the site of collection. Rainy season (July – November) was the best time to collect betel leaves for hydrodistillation.

RESULTS

Hydrodistillation Experiments for Supporting Enzymatic Reactions

(a) *Hydrodistillation of betel oil in deionized water.* 1 gram of betel essential oil was added to 1000 ml of deionized water (pH = 6.8) and submitted to hydrodistillation. Oil was recovered almost quantitatively and showed on GC-MS chromatogram no significant change in the composition of the phenolic components. No APC was found in the water remaining in the flask.

(b) *Variation of the percentages of the phenolic components in betel oil with different modes of leaf pretreatment before hydrodistillation.* Hydrodistillation was performed with betel leaves according to **Hydrodistillation of Betel Leaf Essential Oil** (as above). Results of the hydrodistillation were recorded in Table 3.

(c) *Example of calculation concerning the transformation of **2** and **3** into **4**.* By comparison of *cases 3* and *4*, increase of **1** was due to the partial hydrolysis of **2** and to the transformation of **3**. The latter was hydrolyzed to give **4** which was then partially methylated in para position**.** The unmethylated APC **4** remained in the water in the distilling flask:

- Total increase of **1**: 205.71 – 37.07 = 168.64 mg
- Total decrease of **3**: 1016.26 – 36.99 = 979.28 mg

Table 2. Composition of the phenolic compounds in betel oil after hydrodistillation.

Experiment	Betel oil sample	Weight of oil (mg)	**1** (% Area GC-MS)	**2** (% Area GC-MS)	**3** (% Area GC-MS)
	Betel oil before distillation	1000	6.72	18.10	62.87
1	Betel oil after distillation (Starting temperature: 30°C)	969	6.43	17.21	62.20
2	Betel oil after distillation (Starting temperature: 70°C)	951	6.35	17.74	60.04

Table 3. Oil content and weights of **1**, **2**, **3**, **4** according to leaf pretreatment modes.

Experiment	Operating conditions	Oil content (Mg)	**1,** mg (% area GC-MS**)**	**2,** mg (% area GC-MS**)**	**3,** mg (% area GC-MS**)**	APC **4,** mg (GC-MS)
3	Hydrodistillation with unsalted water, starting temperature: 30°C	513	205.71 (34.16)	55.01 (16.43)	36.99 (6.58)	904.55
4	Hydrodistillation with saturated NaCl solution, starting temperature: 30°C	1270	37.07 (3.16)	138.60 (17.10)	1016.26 (68.37)	367.78

- Increase of **1** from hydrolysis of **2**: (138.60 – 55.01) × 164/206 = 66.54 mg
- Increase of **1** from **3**: 168.64 – 66.54 = 102.10 mg
- Weight of **3** which gave **1**: 102.10 × 234/164 = 145.67 mg
- Weight of **3** which gave **4**: 979.28 – 145.67 = 833.61 mg
- Corresponding weight of **4** from hydrolysis of 833.61 mg of **3** = (833.61 × 150)/234 = 556.12 mg. This quantity would correspond to the increase of **4** in *Experiment 3* by comparison with *Experiment 4*. The experimental value was: 904.55 – 367.78 = 536.77 mg of APC, in reasonably good agreement with the calculated value.

(d) *Results concerning the transformation of **2** and **3** into **4**, derived from Table 3* **(**Table 4). Comparison of cases 3 and 4 showed that increase of chavibetol **1** came mainly from the decrease of 3 and to a lesser extent directly from hydrolysis of chavibetol acetate **2**.

(e) *Hydrodistillation in the presence of $MgCl_2.6H_2O$.* Betel leaves (400 grams) were homogenized in 1000 ml of water containing 3 grams of $MgCl_2.6H_2O$, let at room temperature for 1 h before hydrodistillation for 2 h, then saturated with NaCl and hydrodistilled for one more hour (Table 5). More compound **1** would be obtained concurrently with a decrease of compound **4**.

Similar calculations provided the results in Table 6.

Results showed that in the presence of Mg^{++}, further methylation of **4** occurred, leading to a substantial increase of oil yield with a much larger content of **1** in the essential oil. Besides, Mg^{++} was originally found in fresh betel leaves (1100 mg/kg). These facts supported the existing action of the enzyme catechol O-methyl transferase in betel leaves.

(f) *Hydrodistillation in water containing APC.* To provide more support to the enzymatic intervention, hydrodistillation was carried out with betel leaves ground in water containing already APC from a previous batch (Table 7):

In principle, if there was no further methylation of the pre-existing APC from *Experiment 7*, the sum of APC recovered in the water after hydrodistillation in *Experiment 9* would be the sum of APC in *Experiments 7* and *8* (911.72 + 970.07 = 1881.79 mg). In fact two events occurred:

- Methylation of the preexisting APC indeed took place to give 249.58 – 149.71 = 99.86 mg chavibetol coming from methylation of 91.34 mg of APC.
- Less hydrolysis of APC diacetate **3** to the extent 133.01 – 53.56 = 79.45 mg, leading to a decrease of 50.93 mg of APC from the

Table 4. Calculated results of transformation obtained from Table 3.

Comparison	Increase of **1,** mg	Decrease of **2,** mg	Decrease of **3,** mg	**1,** mg) (Formed from **2**)	**1,** mg (Formed from **3**)	**3,** mg (Producing unreacted **4**)	Unreacted **4,** mg (Calculated)	Unreacted **4,** mg (Found)
3 and **4**	168.64	83.59	979.28	66.54	102.10	833.61	556.12	536.77

Table 5. Results of hydrodistillation in the presence of $MgCl_2.6H_2O$.

Experiment	Pretreatment of leaves before hydrodistillation	Oil content (mg)	**1,** mg (% area GC-MS**)**	**2,** mg (% area GC-MS**)**	**3,** mg (% area GC-MS**)**	**APC 4,** mg (GC-MS)
5	Ground leaves dipped in unsalted water for 1 h, hydrodistillation for 2 h, then saturation with NaCl and hydrodistillation continuing for 1 more hour	508	151.66 (24.70)	35.33 (12.91)	16.73 (2.59)	718.78
6	Ground leaves dipped in water containing $MgCl_2.6H_2O$ for 1 h, hydrodistillation for 2 h, then saturation with NaCl and hydrodistillation continuing for 1 more hour	598	277.64 (37.08)	27.62 (10.23)	8.09 (1.05)	623.83

Table 6. Increase of methylation of **4** in the presence of $MgCl_2.6H_2O$.

Comparison	Increase of **1**, mg	Decrease of **2**, mg	Decrease of **3**, mg	**1,** mg (From decrease of **2** and **3)**	**1,** mg (From more methylation of **4**)	Decrease of **4** because of more methylation, giving 113.78 mg of **1**, mg (Calculated)	Decrease of **4**, mg (Found)
Cases 5 and 6	125.98	7.71	8.64	12.20	113.78	104.07	94.95

Table 7. Results of hydrodistillation in water containing APC.

Experiment	Operating conditions	Oil content (mg)	**1,** mg (% area GC-MS**)**	**2,** mg (% area GC-MS**)**	**3,** mg (% area GC-MS**)**	**APC 4,** mg (GC-MS)
7	Hydrodistillation with unsalted water for 3 h, starting temperature: 30°C	430	112.50 (26.66)	4.54 (8.95)	4.85 (2.14)	911.72
8	Hydrodistilation wth unsalted water for 2 h then saturation with NaCl and hydrodistillation continuing for one more hour, starting temperature: 30°C	512	149.71 (28.55)	57.29 (17.54)	53.56 (11.38)	970.07
9	Hydrodistillation with water remaining in the distilling flask from Experiment *7* for 2 hours, then saturation with NaCl and hydrodistillation continuing for one more hour, starting temperature: 30°C	685	249.58 (32.94)	69.99 (16.12)	133.01 (13.12)	1727.12

total sum of 1881.79 mg. The theoretical total amount of APC recovered from *Experiment 9* should be therefore 1881.79 – (50.93+ 91.34) = 1739.52 mg. This value was in relatively good agreement with the experimental figure of 1727.12 mg of APC. The very small difference in hydrolysis of **2** into **1** in 2 *Experiments 8* and *9* might be neglected for simplification of calculation (Table 8).

Also the increase in oil content must simply come from the increase of 99.86 mg of hydrodistilled chavibetol **1** and 79.45 mg of non-hydrolyzed **3**. This increase of 99.86 mg + 79.45 mg = 179.31 mg was in good agreement with the experimental value of 173 mg for oil. Thus the calculated results for APC fitted quite well with the experimental values from: (b) *Variation of the percentages of the phenolic components in betel oil with different modes of leaf pretreatment before hydrodistillation,* to: (d) *Hydrodistillation in water containing APC,* above.

Determination of Experimental Conditions for Better Yield of Betel Leaf Essential Oil with Higher Content of Total Phenolic Compounds and of APC Diacetate 3

Process optimization was obtained with Statgraphics Centurion XVI.II. First of all, results found for different experimental conditions of hydrodistillation of Hocmon betel leaves according to heading: Hydrodistillation of Betel Leaf Essential Oil, above were recorded in Table 9.

Mutiple variable analysis by Statgraphics Centurion XVI.II gave the following correlation table between each pair of variables (Table 10).

Table 8. Increase of methylation with hydrodistillation in water containing APC.

Oil increase (mg)	Increase of **1** (mg)	Corresponding decrease of **4** (mg)	Decrease of **4** due to less hydrolysis of **3** (mg)	Theoretical sum of APC in *Experiment 7* and *8* (mg)	APC in *Experiment 9* (mg) (Calculated)	APC in *Experiment 9* (mg) (Found)
173	99.86	91.34	79.45	1881.79	1739.52	1727.12

Table 9. Experimental results relative to betel leaf hydrodistillation.

Experiment	Oil content (mg)	Chavibetol (% are GC-MS)	Chavibetol acetate (% are GC-MS)	APC diacetate (% are GC-MS)	Total phenolic compounds (%)
30°C, no salt	0.512	29.20	16.62	11.61	57.43
30°C, no salt	0.503	28.55	17.54	11.38	57.47
30°C, 36% NaCl	1.307	2.46	16.57	74.07	93.10
30°C, 36% NaCl	1.276	2.22	15.43	75.10	92.75
70°C, no salt	0.551	28.07	15.55	10.21	53.83
70°C, no salt	0.547	28.4	14,99	12.11	55.50
70°C, 36% NaCl	1.394	2.56	15.06	74.74	92.36
70°C, 36% NaCl	1.408	2.09	14.28	75.99	92.36
50°C, 18% NaCl	0.942	6.95	21.72	45.34	74.01
50°C, 18% NaCl	0.939	7.88	21.58	44.99	74.45
50°C, 18% NaCl	0.951	6.71	20.82	46.99	74.52

Table 10. Correlation between pairs of variables.

Correlation

	Betel oil	Chavibetol	Chavibetol acetate	APC diacetate	Total phenolic	Temperature	Salt
Betel oil		–0.9476 (11) 0.0000	–0.1475 (11) 0.6652	0.9944 (11) 0.0000	0.9899 (11) 0.0000	0.0918 (11) 0.7884	0.9946 (11) 0.0000
Chavibetol	–0.9476 (11) 0.0000		–0.1625 (11) 0.6311	0.9618 (11) 0.0000	–0.9464 (11) 0.0000	–0.0119 (11) 0.9724	–0.9494 (11) 0.0000
Chavibetol acetate	–0.1475 (11) 0.6652	–0.1625 (11) 0.6311		–0.0983 (11) 0.7737	(11) 0.7170	–0.2525 (11) 0.4538	–0.1351 (11) 0.6921
APC diacetate	0.9944 (11) 0.0000	0.0918 (11) 0.0000	–0.0983 (11) 0.7737		0.9973 (11) 0.0000	0.0035 (11) 0.9919	0.9987 (11) 0.0000
Total phenolic	0.9899 (11) 0.0000	–0.9464 (11) 0.0000	–0.1237 (11) 0.7170	0.9973 (11) 0.0000		–0.0457 (11) 0.8939	0.9982 (11) 0.0000
Temperature	0.0918 (11) 0.7884	–0.0119 (11) 0.9734	–0.2525 (11) 0.4538	0.0035 (11) 0.9919	–0.0457 (11) 0.8939		0.0000 (11) 1.0000
Salt	0.9946 (11) 0.0000	–0.9494 (11) 0.0000	–0.1351 (11) 0.6921	0.9987 (11) 0.0000	0.9982 (11) 0.0000	0.0000 (11) 1.0000	

Correlation
(Sample Size)
P-value

The calculated correlation between pairs of variables (Table 10) showed strong correlation:

- Between betel oil content and the percentages of chavibetol, APC diacetate, total phenolic compounds and salt
- Between chavibetol percentage and betel oil content, the percentages of APC diacetate, of total phenolic compounds and salt
- Between APC diacetate percentage and betel oil content, the percentages of chavibetol, of total phenolic compounds and salt
- Between total phenolic compound percentage and betel oil content, the percentages of chavibetol, APC diacetate and salt; and
- Between salt percentage and betel oil content, the percentages of APC diacetate and of total phenolic compounds.

No significant correlation was observed between temperature and betel oil content, the percentages of chavibetol, chavibetol acetate, APC diacetate , salt and of total phenolic compounds (P-value much higher than 0.05).

With two experimental factors: temperature (range 30°C – 70°C), salt (range 0% – 36%) and the response variables: oil content (mg), total phenolic content (%), APC diacetate (%), the designing class: screening, the process factors model: two-factor interactions, the analysis of variance gave the following results (Table 11):

Table 11. Results of analysis of variance.

Parameters	P-values			R^2 (%)
	A: Temperature	B: Salt	AB	
Oil content	0.0001	0.0000	0.0053	99.94
Total phenolic compounds (%)	0.0020	0.0000	0.0135	99.95
APC diacetate	0.8281	0.0000	0.5912	99.88

In accordance with the results of analysis of variance, the regression equations for betel oil content, total phenolic compounds and APC diacetate percentages were obtained by Statgraphics Centurion XVI.II as follows:

- Betel oil content (mg) = 478.216 + 1.0375*(temperature) + 20.3611*(salt) + 0.047222*(temperature)*(salt); r^2 = 0.9994

- APC diacetate percentage (%) = 12.4614 + 1.7293*(salt); r^2 = 0.9975

- Total phenolic percentage (%) = 59.5205 – 0.0696*(temperature) + 0.9477*(salt) + 0.0014*(temperature)*(salt); r^2 = 0.9994

DISCUSSIONS

When starting hydrodistillation at 30°C, oil yield, 4-allylpyrocatechol diacetate and the total phenolic compounds percentages were highest with hydrodistillation of fresh betel leaves in saturated NaCl solution. Calculations showed that in unsalted water, hydrodistillation led to a substantial increase of **1,** due in greater part to the conversion of **3** by hydrolysis into **4**, followed by a partial selective O-methylation of OH in para position to the allyl group. NaCl appeared to refrain to a great extent this conversion reaction.

Conversion of **3** into **1** increased substantially with hydrodistillation performed in the presence of Mg^{++}.

Hydrodistillation of betel leaves in water containing APC showed that methylation took place even with free APC.

No such reaction occurred when betel oil was submitted to hydrodistillation in DI water without the presence of betel leaves.

All these experimental facts were in favour of enzymatic reactions leading to conversion of **2** and a greater part of **3** into **1** according to the above suggested mechanism.

Optimization study with Statgraphics Centurion XVI.II revealed the following conclusions:

- Temperature factor affected only slightly the oil content and the total phenolic percentage and had no significant effect on APC diacetate percentage.

- Salt was found to be the main factor influencing oil yield, percentages of phenolic compounds and APC diacetate. Therefore, to reduce technical difficulties and operational cost in pilot production as well as in industrial production, it is recommended to perform the hydrodistillation starting at room temperature in saturated NaCl water, in this case the yield of oil, APC diacetate and of total phenolic content reach practically the maximum values.

CONCLUSION

For the first time, enzymatic reactions in the betel leaves were revealed and strongly supported by concrete experimental facts. Salt strongly refrained the enzymatic action and increased the oil yield as well as the total phenolic content and APC diacetate. As temperature influences very weakly on oil yield and total phenolic

content, it was strongly recommended to perform hydrodistillation in saturated NaCl water starting at around 30°C to get better quality oil of high yield with high content of phenolic compounds and APC diacetate, for less intense labour, time expense and cost effectiveness.

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