Research Journal of
Phytochemistry

ISSN 1819-3471

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Phytochemistry 8 (4): 188-198, 2014
ISSN 1819-3471 / DOI: 10.3923/rjphyto.2014.188.198

Phytochemical Study of the Constituents of *Napoleona imperalis* and It's Flavourant Properties

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ABSTRACT

Most spices, owe their individual properties to the pharmacologically active secondary plant metabolites that they contain. In this study, five solvent extracts (hexane, acetone, ethylacetate, ethanol and water) obtained from *Napoleona imperalis*, P. Beauv. (Lecythidaceae) known as Ntum in Akaeze and Ikwuano dialect of Igbo language of Nigeria, traditionally used as local sweetener, were evaluated for its flavourant properties. The presence of major phytoconstituents was detected qualitatively, the results of the cold and hot solvent extraction revealed that Ethanol (ETE) gave the highest percent extract recoveries of 4.17 and 1.81% for hot and cold extraction methods, respectively. The flavour threshold was determined at neutral (7.0) and slightly acidic (4.5) pH, respectively. Results revealed that extracts of ETE and water (WAE) showed strong presence of the active sweet compound up to 0.1%. At pH 7.0 but at pH 4.5 the extracts of ETE and WAE showed strong presence of the active sweet compound up to 0.01%. Sensory attribute depicts that mean sensory scores for test samples 397 and 460 had the same scores for sweetness (4.2±0.23) while sample 222 had a score of 4.1±0.5 with the lowest score of 3.7±0.11 going to sample 760. No significant difference was observed between these values except for sample 760 at $p<0.05$.

Key words: Phytochemicals, sweetener, flavour threshold, *Napoleona imperialis*

INTRODUCTION

Biologically active compounds present in plants have always been of great interest (Ibrahim *et al*., 2012; Roopashree *et al*., 2008; Obasi *et al*., 2010) and the value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladun *et al*., 2007). Most spices, condiments, teas and other beverages, owe their individual properties to the pharmacologically active secondary plant metabolites that they contain (Okwu, 2001). Flavourings are substances used to give taste and/or smell to food and are classified according to legislation as natural, nature-identical or artificial flavouring substances (Li *et al*., 2002). Taste is the combined sensations arising from specialized taste receptor cells located in the mouth. It is primarily limited to the tongue and is broken down into the sensations of sweet, sour, salty, bitter and umami (Lindemann *et al*., 2002). Umami has intuitive appeal because the archetypal stimuli for umami is glutamic acids (Breza *et al*., 2007; Lindemann *et al*., 2002). Glutamate and free fatty acids are reported to elicit sensations in humans and rats that are qualitatively different from the four classic primary tastes (Lindemann *et al*., 2002;

McCormack *et al.*, 2006; Stratford *et al.*, 2006). Monosodium glutamate (MSG) and fat often are used as food additives to increase flavour and palatability and unlike the other four flavours, umami is not thought to have a flavour of its own but rather it intensifies other flavours already present, hence the common use of MSG in many pre-packaged food items (Yamaguchi and Kimizuka, 1979; Pyke, 1970).

Despite the seemingly limited combination of flavour properties, extremely large numbers of flavours are able to be sensed by the tongue. Less is known about sweet substance and there seems to be no relationship between organic functional groups and sweet flavour, as almost every class of compound has a member which tastes sweet, yet at the same time the chemistry is very specific (Heyer *et al.*, 2003). Natural flavours are those which are produced by nature or through an engineered biological reaction (Mosel and Kantrowitz, 1952) while artificial flavours are created by man through chemical reactions. Natural flavours are not simply one or two compounds which could be easily synthesized but a large number of compounds in a characteristic distribution, called a flavour profile (Lawless and Heymann, 1999).

Glycosidically bound compounds have been identified in a vast number of fruits (Williams *et al.*, 1980, 1982a, b; Sefton *et al.*, 1994) but the knowledge about the glycosides occurring in strawberries is not so extended, except for the 2,5-dimethyl-4-hydroxy-3(2H) furanone (furaneol glucoside) that has been largely studied (Mayerl *et al.*, 1989) Glycosides containing a polycyclic aglycone moiety of either C_{27} steroid or C_{30} triterpenoid (collectively known as sapongenins) attached to a carbohydrate have been reported in *Napoleona imperalis* seed (Ukpabi and Ukpabi, 2003).

Though, *N. imperialis* is one of the lesser known plants, its economic importance has partially been reported by Dalziel (1955) and Irvine (1961). These include the use of the fruits sugary pulp as desserts, the roots for medicinal purposes and the twigs as traditional chewsticks (Ukpabi and Ukpabi, 2003). The plant is commonly known as Ntum in the Akaeze and ikwuano dialect of Igbo language of Nigeria and is traditionally used as local sweetener.

Taking into consideration the economic importance of this plant, five different solvents (ethanol, acetone, hexane, ethylacetate and water) extract of fruit of *Napoleona imperialis* were analyzed for organoleptic properties. This study will help to identify the component with flavourant properties.

MATERIALS AND METHODS

Research duration: This study was carried out at the Chemical Science Laboratory, Evangel University Akaeze from 2010-2014.

Collection and identification of plant materials: Ripened fruit of *Napoleona imperalis* was collected from uncultivated farmland located at Southern parts of Nigeria. The plant sample was identified and authenticated at Biological Science Department herbarium, Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State, Nigeria.

Sample preparation and extraction procedure: The fresh ripened fruit pulp was ground into fine slurry using a blending machine. Hexane (40-60) (Merck, India), ethyl acetate (Merck, India), acetone (Merck, India), ethanol (Merck, India) and water was employed for the extraction of the plant's active principles. For the hot solvent extractions, 150 g of the ground plant material was wrapped in Whatman filter paper, each wrap containing 25 g were placed in a thimble of a Soxhlet

apparatus. Then 750 mL of each solvent added into the round bottom flask and Soxhlet apparatus mounted. The round bottom flask was heated and extraction of the plant material carried outand then stopped after several refluxes. The solution was evaporated to dryness using a rotary evaporator (model type 34/2; Corning Ltd, England). Percentage yield obtained in relation to the dry material calculated.

For the cold solvent extractions, 150 g of the ground plant sample was weighed into 750 mL of each solvent, hexane, ethanol (95%), ethyl acetate, acetone and water in a conical flask. It was covered, shaken every 1 h 30 min, for 6 h and thereafter, allowed to stand for 72 h for extraction. The solution at the end of extraction was shaken and filtered using Whatman filter paper No 45. The filtrate was subsequently evaporated to dryness using a rotary evaporator and percentage yield obtained in relation to the dry plant material calculated. The extracts obtained were stored at ambient temperature in amber coloured bottles until required.

Phytochemical screening: Simple but rapid qualitative chemical tests to detect the presence or otherwise of alkaloids, saponins, glycosides, terpenoids and other secondary metabolites was carried out on the plant crude extracts using the procedure outlined by Harborne (1973), Sofowora (1993) and Trease and Evans (1989).

Test for alkaloids: About 0.4 g extract of each sample was mixed with 8 mL of 1% HCl, warmed and filtered. Two milliliter of each filtrate was titrated separately with Mayer's reagent and Dragendroff's reagent. Turbidity of precipitation was observed to indicate the presence of alkaloids. Wagner and hagers regaents all gave reddish brown and yellow coloured precipitate while alkaloids yields buff colour precipitate in 10% tannic acid.

Test for glycosides: The extracts were hydrolyzed with mineral acid and then tested for the glycone and aglycone moieties. Raymond test gave violet colour with dinitrobezene in hot methanolic alkali and legal test yielded red colour when extract was treated with sodium nitroprusside. Test solution when treated with bromine water gave yellow precipitate.

Saponin test: About 20 mg of the each sample was boiled in 20 mL of distilled water in a water bath for 5 min and filtered. Then, 10 mL of the each filtrate was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Hemolytic test: Three cups of blood agar was scooped from a blood agar plate using a test tube. Each agar cups were marked and inverted over the agar plates. With the help of a pipette each of the extract was added to agar cups till full and control was set by adding distilled water into the cup. It was allow to stand for an hour and observed for zone of clearing with the three agar cups. Hemolytic zone, positive result indicate rupture of red blood cells.

Test for anthraquinone glycosides: About 200 mg of each plant samples were boiled with 6 mL of 1% HCl and filtered. The filtrate was shaken with 5 mL of benzene. The layer was removed and then 10% NH_4OH was added. Formation of pink, violet or red colour in the alkaline phase was observed for the presence of anthraquinone.

Borntrager test: One gram of alcoholic extract of each sample and allow to evaporate into dryness. The residue was added to 10 mL of distilled water and filtered. Extract the filtrate with 2 mL of benzene extracts into two portions. Dividing the two combined benzene extracts into two equal portions. Reserve one as control. To the second portion add 5 mL of ammonia solution, then shake. Observe the alkaline layer for colour changes. The presence of red colour indicates that anthraquinone is present.

Modified Borntrager test: Evaporate an equivalent of one gram extract to the samples to dryness using a water bath. The residue is mixed with 10 mL of 0.5 N KOH and 1 mL of diluted (5%) hydrogen peroxide with stirring. Heat on a water bath for 10 min, allow to cool and filter. Discard the residue and add glacial acetic acid in drops until the filtrate get acidic and transform blue litmus to red. Extract with 5 mL benzene, two times. Divide the combined benzene extracts into two portions. Reserve one portion as control. Two to 5 mL of ammonia solution is to be added on second portion until alkaline. Observe the alkaline layer for colour changes. Pink colour depicts the presences of anthraquinones.

Test for cyanogenic glycosides: Add 5 g of plant sample in a test tube, moisten with water and add a few drops of Chloroform to enhance enzyme activity. One milliliter of 1% emulsion solution was added to ensure hydrolysis of the glycoside. A firm stopper on the tube was used to stopper the cork which suspended a piece of picrate paper. The paper strip must not be touched the inner sides of the test tube. The tube warmed at 37°C and colour changes was observed on the paper. The appearance of red colour within 15 min is a measure of relative concertration of cyanogenic glycosides. If no colour is observed after 3 h, absence of glycoside is indicated.

Test for cardiac glycosides

Kedde's test: Extract the samples with chloroform, evaporate to dryness, add one drop of 90% alcohol and 2 drops of 2% 3,5-dinitro benzoic acid. Make alkaline with 20% sodium hydroxide solution. A purple colour is produced. The colour reaction with 3, 5-diinitrobenzoic acids depend upon the presence of β-unsaturated O-lactones in the aglycone.

Keller killiani test (test for Deoxy sugars): Extract the samples with chloroform and evaporate it to dryness. Add 0.4 mL of glacial acetic acid containing a trace amount of ferric chloride. Transfer to a small test tube; add carefully 0.5 mL of concentrated sulphuric acid by the side of the test tube, brown ring colour at the inter phase which signifies the presence of cardiac glycoside.

Test for sterols and triterpenoids

Libermann-buchard test: Extract was treated with few drops of acetic anhydride, boil and cool, concentrated sulphuric acid is added from the sides of the test tube, shows a brown ring at the junction of two layers and the upper layer turns green which shows the presence of steroids and formation of deep red colour indicates the presence of triterpenoids.

Salkowski test: Treat extract in chloroform with few drops of concentrated sulphuric acid, shake well and allow standing for some time, red colour appears at the lower layer indicates the presence of Steroids and formation of yellow coloured lower layer indicates the presence of triterpenoids.

Test for carbohydrates

Molisch's test: Treat the test solution with few drops of alcoholic alpha napthol. Add 0.2 mL of concentrated sulphuric acid slowly through the sides of the test tube, a purple to violet colour ring appears at the junction.

Benedict's test: Treat the test solution with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and upon boiling on water bath, reddish brown precipitate forms if reducing sugars are present.

Camnelisation test: Carbohydrates when treated with strong sulphuric acid, they undergo charring with the dehydration along with burning sugar smell.

Selwinoff's test (standard test for ketone): Hydrochloric acid reacts with ketose sugar to form derivatives of furfuraldehyde which gives red coloured compound when linked with resorcinol. Add compound solution to about 5 mL of reagent and boil. Fructose gives red colour within half minute. The test is sensitive to 5.5 mmol L^{-1} if glucose is absent but if glucose is present it is less sensitive and in addition of large amount of glucose can give similar colour.

Fehling's test: Equal volume of Fehling's A (Copper sulfate in distilled water) and Fehling's B (Potassium tartarate and Sodium hydroxide in distilled water) reagents are mixed and few drops of sample is added and Boiled, a brick red precipitate of cuprous oxide forms, if reducing sugars are present.

Determination of flavour threshold (sweetness): This was determined according to the method of Van Cott *et al.* (1954). About 90 mL of distilled water measured into a paper cup and 10 g of sample added until dissolved to yield a 10% (w/w) sample solution. The mouth was rinsed with plain tap water and a clean cotton swab dipped into the 10% solution and smeared around the tongue. Taste with sweetness, was indicated by a + in a data table for 10% sample extract. Other observations were noted. Now measure out 10 mL of the 10% sample solution into a clean paper cup and add 90 mL of distilled water to dissolve to give a 1% sample solution. Again rinse your mouth with plain tap water and dip a clean cotton swab into the 1% solution and smear around your tongue. Taste with sweet note was also indicated by a + in the data table for 1% sample. Note any other observations that you make. Serial dilution was continued and each new solution tested with the cotton swab procedure. The lowest concentration at which one can still taste the sweetness is its approximate taste threshold. This experiment was repeated with, sucrose, aspartame and monosodium glutamate.

Sensory evaluation: Seven panellists were used to perform the sensory evaluation. The panelists were selected on the basis of their interest and availability. Two training sessions conducted in which the panellists was trained to evaluate sensory attributes of the samples. Sensory quality attributes were evaluated using a 5-point scale (Potter and Hotchkiss, 1996) for the specific sensory parameters with 1 for fairly sweet to 5 for extremely sweet for each attribute. The samples were evaluated for sweetness, mouth feel and general acceptability (Amerine *et al.*, 1965). The extracts were evaluated within 24 h after sample collection, cooled and stored at refrigeration temperature (~4°C) until subjected to sensory analysis. Randomly coded samples with control was presented to

the panelists on a glass cup. Samples served to panelists in a room with an overhead fluorescent light. The panelists were instructed to rinse their mouth with tap water before starting and between sample evaluations.

RESULTS AND DISCUSSION

Extraction: The results of the cold and hot solvent extraction of the fruit of *Napoleona imperalis* are presented in Table 1. Ethanol gave the highest percent extract recoveries of 4.17 and 1.81% for hot and cold extraction methods, respectively while acetone gave the least with 2.03 and 0.50% for hot and cold extraction methods, respectively.

Qualitative phytochemical studies of *Napoleona imperalis* fruit was performed on its cold and hot hexane, ethyl acetate, acetone, ethanol and water extracts to identify its alkaloid, steroids and tritepenes, steroidal rings and glycosides by using suitable chemicals and reagents (Table 2-5). Alkaloids were observed absent in all the solvents except in water extract. Steroids, triterpenes and steroidal rings were also observed to be absent in both cold and hot acetone and water crude extract particularly steroidal rings which were not detected in water extract. However, they were observed to be present in ethyl acetate, ethanol and hexane extracts. Qualitative phytochemical studies of carbohydrate and glycoside showed a good characteristic colour and precipitate in all four tested reagent except hexane extract. However, cold acetone extract yielded negative in molisch, monosaccaride and caramelization test. Slight presence of saponin was confirmed by foam in all extracted solvents as saponin glycoside. Phytochemicals are mostly observed present in ethyl acetate, ethanol and water crude extract with strong presence of saponin in the ethanol extract (Chindo *et al*., 2003). Glycosides particularly saponin and anthraquinone glycosides were also observed in extracts. The above qualitative phytochemical screening showed that this plant is a rich source of glycosides. However, presence of steroids, triterpenes and alkaloids is limited in plant samples.

Flavour threshold value of extracts of *N. imperalis* at neutral pH is as depicted in Table 6. Result revealed that extracts of ETE and WAE showed strong presence of the active sweet compound up to 0.1%. This is comparable to those of standard monosodium glutamate and aspartame but greater than the values for sucrose which gave last indication at 1.0% dilution. Extracts of HXE, EAE and ACE gave all negative results. However, aspartame showed the presence of the compound even at 0.01%. This may be because of its level of purity. The organoleptic properties of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol) and 4-methoxy-2,5-dimethyl-3(2H)-furanone (mesifuran) as well as their importance for fruit flavours are well documented (Werkhoff *et al*., 1998) and both compounds probably influence the aroma profile of passion fruit flavour due to their low aroma thresholds (Werkhoff *et al*., 1998).

Table 1: Crude and percentage recovery of cold and hot solvent extract 15.0 g of *Napoleona imperalis*

	Crude recovery weight (g)		Percentage recovery	
Solvent	Cold	Hot	Cold	Hot
Hexane	1.31	3.43	0.873	2.28
Ethyl acetate	1.76	4.79	1.17	3.19
Acetone	0.76	3.05	0.50	2.03
Ethanol	2.72	6.26	1.81	4.17
Water	2.14	4.21	1.42	2.80

Table 2: Phytochemical screening for glycosides in *Napoleona imperialis* crnde solvent extract

				Sapouin glycoside		Anthraquinone glycoside test			Cardiac glycoside		Cyanogenetic
Extracts	Raymond's test	Legal's test	Bromine water test	Froth	Hemolysis	Borntragar'	Modified Boratragar		Kedde's test	Keller killani test	glycoside
Hexane											
Cold	-	-	-	-	-	-	-		-	-	-
Hot	-	-	-	-	-	+	-		-	-	
Ethyl acetate											
Cold	+	+	+	++	+	+	+		+	+	+
Hot	++	+	++	+++	++	+	+		+	+	
Acetone											
Cold	+	+	+	++	++	++	+		+	+	+
Hot	+	+	+	++	++	+	+		+	+	
Ethanol											
Cold	+	+	+	+++	+++	++	+		++	++	++
Hot	+	+	+	+++	+++	++	++		++	++	
Water											
Cold	+	+	+	++	++	+	+		+	+	++
Hot	+	+	+	+	++	+	+		+	+	

Table 3: Phytochemical screening for alkaloids in *Napoleona imperalis*

Extracts	Mayer's test	Dragendorff's test	Wagner's test	Hager's test	Tannic acid test
Hexane					
Cold	-	-	+	-	-
Hot	-	-	-	-	-
Ethyl acetate					
Cold	-	-	-	-	+
Hot	-	-	-	-	-
Acetone					
Cold	-	-	-	-	-
Hot	-	-	-	-	-
Ethanol					
Cold	-	-	-	-	-
Hot	+	+	+	+	-
Water					
Cold	+	-	+	+	+
Hot	++	++	+	++	++

++: Very strong, +: Strong, -: Not detected

Table 4: Phytochemical screening for steroids /triterpenes and steroid rings in *Napoleona imperalis* crude extracts

Extracts	Steroids	Triterpenes	Steroidal rings
Hexane			
Cold	+	+	+
Hot	++	++	+
Ethyl acetate			
Cold	+	+	+
Hot	+	+	+
Acetone			
Cold	-	-	-
Hot	-	-	-
Ethanol			
Cold	+	+	+
Hot	++	+	+
Water			
Cold	-	-	nd
Hot	+	+	nd

++: Very strong, +: Strong, -: Not detected, nd: Not determined

Table 6, revealed the sweetness threshold of five extracts of *Napoleona imperalis* fruit at pH 4.5. The results depict that ETE and WAE extracts show strong presence of the active sweet property up to 0.01%. This is comparable to those of standard, monosodium glutamate and aspartame but greater than those values for sucrose which gave last indication at 0.1% dilution. Buttery *et al.* (1997) has shown that the threshold value in water is pH dependant. Research has also shown that xylitol a natural sweetener found in various berries reduces levels of mutant streptococci bacteria (FSA., 2010; Brandle, 2004; FAO and WHO., 2000).

Sensory evaluation: The mean sensory scores of controls and samples are presented in Table 7. Test samples 397 and 460 had the same scores for flavour (4.2±0.27) while sample 222 had a score of 4.1±0.5 with the lowest score of 3.7±0.11 goes to sample 760. No significant difference was observed between these values except for sample 760 at ($p<0.05$). This is comparable to the result of Yamaguchi and Kimizuka (1979). Also overall acceptability and mouth feel for samples 222, 397

Table 5: Phytochemical screening for carbohydrates in *Napoleona imperalis* crude extract solvent

Extracts	Molisch's test	Bendedict's (monosaccaride test)	Caramelization test	Ketone test	Fehling's test
Hexane					
Cold	-	-	-	-	-
Hot	-	-	-	-	-
Ethyl acetate					
Cold	+	+	+	+	+
Hot	+	+	+	+	+
Acetone					
Cold	-	-	-	+	+
Hot	+	+	-	+	+
Ethanol					
Cold	+	+	++	++	+
Hot	++	+	++	++	+
Water					
Cold	+	+	+	+	+
Hot	+	++	++	+	+

++: Very strong, +: Strong, -: Not detected, nd: Not determined

Table 6: Taste (sweetness) threshold for *Napoleona imperalis* extracts expressed in percentage w/w at pH 7 and 4.5

	10	1	0.1	0.01	0.001
Substance extracts	----------(%)----------				
At pH 7					
HXE	-	-	-	-	-
EAE	-	-	-	-	-
ACE	-	-	-	-	-
ETE	++	++	++	-	-
WAE	++	+	++	-	-
Control sucrose	++	-	-	-	-
MSG	++	++	++	-	-
Aspartame	++	++	++	+	-
At pH 4.5					
HXE	-	-	-	-	-
EAE	-	-	-	-	-
ACE	-	-	-	-	-
ETE	++	++	++	+	-
WAE	++	++	++	+	-
Sucrose MS	++	+	-	-	-
Glutamate	++	++	++	+	-
Aspartame	++	++	++	++	+

++: Very strong , +: Strong , -: Not detected

Table 7: Sensory profile of water extract of *Napoleona imperalis* and experimental controls

	Samples			
Sensory attribute	222	397	460	760
Sweetness	4.1±0.50	4.2±0.27	4.2±0.23	3.7±0.11
Mouth feel	3.4±0.22	3.1±0.31	3.4±0.56	2.8±0.44
General acceptability	4.0±0.01	4.0±0.13	4.0±0.52	1.6±0.47

and 460 have same rating except for sample 760 which had a low rating of 1.6±0.47 and 2.8±0.44. These result showed that sample 397 has high sensory attributes comparable to those sweeteners in the market.

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