UJMR, Volume 3 Number 2, December, 2018 *ISSN: 2616 - 0668*
https://doi.org/10.47430/ujmr.1832.017
Received: 10/12/2018 **Accepted:** 15/01/2019

Evaluation of Synergistic Activity of Black Seed Extract (*Nigella sativa*), Honey and Zamzam Water on *Candida albicans*

***Ummasalma Aliyu Saulawa[1, 2] and Sani Liti Hamisu[1]**

1. Department of Microbiology, Faculty of Natural and Applied Sciences, Umaru Musa Yar'adua University Katsina, Nigeria.
2. Institute of Infection and Global Health, School of Life Science, University of Liverpool, United Kingdom.

Contact address: *umsal4uu@gmail.com; ummasalma.aliyu@umyu.edu.ng: 08028985229

Abstract

Pathogenic yeast are resistant to the existing synthetic antifungal agents, demanding an additional efforts to seek more effective therapeutic agents as antifungal against such pathogens. In order to achieve this, we try to evaluate the antifungal activity of black seed, honey and zam-zam water using C. albican as the test organism. Samples of black seed powder, honey (HN) and Zam-zam water (ZZ) were collected from Katsina metropolis, Katsina state. The samples were then transported to the Microbiology laboratory, Umaru Musa Yar'adua University Katsina for analysis. An ethanolic extraction of the black seed was prepared. The clinical isolates C. albicans was cultured on Sabouraud dextrose agar (SDA). Agar well diffusion method was used in evaluating the synergistic activity of the test samples on C. albicans. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of the test samples were also evaluated. The result shows that ethanolic black seed extract (BSE) has the highest zone of inhibition at 14mm followed by honey (HN) with 12mm while Zam-zam (ZZ) water having the least at 2mm. The MIC and MFC of the most active sample were found to be at 30mg/ml and 60mg/ml respectively.

Keywords: *Black seed, Honey, Zam-Zam, Candida albicans*

INTRODUCTION

Candida albicans is a commensal human gut flora as well as opportunistic pathogens of the humans (Erdogan and Rao, 2015). Synergetic activity of antimicrobial agents are effects arising between two or more agents, entities, factors, or substances that produces an effect greater than the sum of their individual effects. Antimicrobial agents are substances that have therapeutic effect on microorganisms. These antimicrobial agent are synthesized chemotherapeutic substances obtained majorly from microorganisms, plants and some animal product (Zarb and Goossens, 2012). Medicinal plants have a long history of use which is widespread in both developing and developed countries (Nafiu, 2017). Compared to antimicrobials that have relatively expressed some side effect such as the rise in resistance of bacteria against antibiotics, medicinal plants and compounds are more tolerant with few side effects, affordable, and acceptable due to long history of therapy and being renewable in nature (Nafiu, 2017). *Nigella sativa* (*N. sativa*) seed is a medicinal plant also known as 'Black Seed', 'Al-Habba Al-Sauda' or 'Al-Habba Al-Barakah' (Ahmad *et al*, 2013). It is also well known in the Middle East, and Middle Asia as a natural remedy for many ailments and as a flavouring agent in bread and prickles (Krishnapura, 2018). An authentic saying of the Prophet Muhammad (Peace Be Upon Him) about black seed is also quoted in Al-Bukhari: Abu Huraira (Allah be pleased with him) narrated that Allah's Apostle (peace be upon him) said "Use the black seed, which is a healing for all diseases except 'As-Sam" and As-Sam is Death (Al-Bukhari. hadith).[1]

The medicinal importance of honey and black seed has been documented in the world's oldest medicinal literatures, and from the ancient times, they have been known to possess antimicrobial properties especially honey in wound healing activity (Mandal and Mandal, 2011). The institute of the Custodian of the Two Holy Masjids for Al Hajj research centre in Om Al-Qura university conducted a special research and examined the extent of purity of Zam-zam water and found that it has a wonderful physique that makes it different from other drinkable water because it is naturally pure and sterile that has no microbes in it (Al Zuhair and Khounganian, 2006).

[1] Sahih Al-Bukhari 5688: Book 71, Hadith11

Black seed

N. sativa is an annual flowering plant. It has an average height of 20-30cm with linear lanceolate leaves. *N. sativa* has an average of 5-10 petals, which are normally either yellow, pink, white, pale blue or purple in coloration (Ahmad *et al*, 2013). The 3-7 united follicles of the fruit contains numerous seeds. The black colored seeds are flattened, oblong and angular, funnel shaped, with the length of 0.2 cm and 0.1 cm wide (Ahmad *et al*, 2013). *N. sativa* is native to Southern Europe, North Africa and Southwest Asia and it is cultivated in many regions in the world such as Middle Eastern Mediterranean region, South Europe, India, Pakistan, Syria, Turkey, Saudi Arabia (Krishnapura, 2018). In the Middle East, Northern Africa and India, it has been used traditionally for centuries for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema and for its antihistaminic, antidiabetic and anti-inflammatory activities (Kumar et al., 2010).

N. sativa has oil and constituents such as thymoquinone (TQ) which has shown potential medicinal properties; they exhibit potent anti-inflammatory effects on several inflammation-based models including experimental encephalomyelitis, colitis, peritonitis, oedama, and arthritis through suppression of the inflammatory mediators prostaglandins and leukotriens (Kumar et al., 2010). Salem, 2005, reported that *N. sativa* has immunogenic effect on T cell and natural killer cell-mediated immune responses. Most importantly, both the oil and its active ingredients expressed anti-microbial and anti-tumor properties toward different microbes and cancers (Khan *et al.*, 2011). More than 150 studies have been conducted and the pharmacological effectiveness of *Nigella sativa* seed constituents have been confirmed, though, *Nigella sativa* seed is a complex substance of more than 100 compounds, some of which have not yet been identified or studied (Salem, 2005).

Honey

Honey use and production has a long and varied history. In many cultures, honey has associations that go beyond its use as a food but also for its therapeutic properties. It is frequently used as a talisman and symbol of sweetness. The spiritual and therapeutic use of honey in ancient India is documented in both the Vedas and the Ayurveda texts, which were both composed at least 4,000 years ago.

In Islam, an entire chapter (Surah) in the Qur'an is called An-Nahl (the Bee). According to the teachings (hadith), Prophet Muhammad (SAW) strongly recommended honey for healing purposes. The Qur'an promotes honey as a nutritious and healthy food "And thy Lord taught the Bee to build its cells in hills, on trees, and in (men's) habitations; Then to eat of all the produce (of the earth), and find with skill the spacious paths of its Lord: there issues from within their bodies a drink of varying colours, wherein is healing for men: verily in this is a Sign for those who give thought" [Al-Quran 16:68-69][2]. In the Christian New Testament, Matthew 3:4, John the Baptist is said to have lived for a long period of time in the wilderness on a diet consisting of locusts and wild honey.

Zamzam water

According to Arab historians, the Zamzam well has been in used for around 4000 years. The well marks the site of spring where Allah, in His mercy, sent the Angel Gabriel, who scraped the ground, causing the spring to appear. That was when Hajar Prophet Abraham's wife, and their infant son Ismail (AS) were in desperate search for water to quench their thirst (Saudi GS, 2013). On finding the spring, and fearing that it might run out of water, Hajar enclosed it in sand and stones. The name Zamzam originates from the phrase of ZomeZome, meaning 'stop flowing'. The Zamzam well is located within the Holy Masjid at about 20m east of the Ka'ba in Makkah (Saudi GS, 2013). In 1971, the Ministry of Agriculture and Water Resources sent samples of Zamzam water for investigation to the European laboratories to test the portability of the Zamzam water. The results of the water samples tested by the European laboratories showed that Zamzam water has a special physique that makes it an advantageous water. The main difference between Zamzam water and other water (city water) is in the quantity of calcium and magnesium salts, the content of these was slightly higher in Zamzam water, but more significantly, the water contains fluoride that have an effective germicidal action. Moreover, the remarks from the European laboratories showed that the water is fit for drinking (Al-Zuhair, and Khounganian, 2006).

The institute of the Custodian of the Two Holy Masjids for Al Hajj research centre in Om Al Qura University also conducted a special research and examined the extent of purity of Zam-zam water and found out that it has a wonderful physique that makes it different from other drinkable water. Because it is naturally pure and sterile with no microbes in it (Al Zuhair and Khounganian, 2006).

[2] Suratul Naml; Chapter 16, verse 68-69.

Biological growth and vegetation usually takes place in most wells. This makes the water unpalatable owing to the growth of algae causing taste and odour problems. But in case of Zamzam well, there wasn't any sign of biological growth (Saudi GS, 2013).

MATERIALS AND METHODS

Culture and Isolation of *C. albicans*

C. albicans was obtained from Microbiology Laboratory Isolates Bank. The isolate was sub-cultured on sabouraud dextrose agar (SDA) and later the isolates undergo a Germ tube test for identification and confirmation as demonstrated by (Raju and Rajjap, 2011). Biochemical test was carried out to further confirm the test isolate (Subhash, 2006)

Preparation of Black Seed Extract

The black seed powder was purchased from Kofar-Kaura Islamic vendors in Katsina. 100g of the powdered seed was weighed, and mixed with 500ml of ethanol. This was agitated for three hours and allow to stand for 24 hours. It was later filtered using a Whatmann filter paper of diameter 125mm and pore size of 0.7mm. The residue were discarded and the filtrate was evaporated at 40^0C to dryness on a water bath to obtain the black seed ethanolic extract (Idris *et al.*, 2016).

Preparation of Varied Concentration of Black Seed Extract (BSE), Honey (HN) and Zam-zam (ZZ)

Stock solution of the test samples (BSE, HN & ZZ) were prepared by dissolving 0.6ml of the samples each into a 5.4ml of distilled water. Serial dilution of the stock solutions were employed to obtain the concentration of 60mg/ml, 30mg/ml, 15mg/ml, 7.50mg/ml, 3.75mg/ml and 1.88mg/ml respectively for each test sample (BSE, HN &ZZ) (CLSI, 2014).

Antifungal Assays for the Effectiveness of Black Seed Extract, Honey and Zam-zam water

Fungal suspension of *C. albicans* was obtained by picking 1-3 colonies of the isolate from an overnight culture and placing it into 5ml saline. The concentration was adjusted to 1-2x10^8 CFU/ml by comparing with the McFarland 0.5 standard in bright light (Arevalo *et al.*, 2003). The fungal suspension was seeded onto a fresh prepared SDA plates. Antifungal activity of the test samples was carried out using agar well diffusion method on SDA. Five wells (4 treatments + 1 control) were punched on each plate using a sterile cork borer and labelled appropriately. 0.5ml of the varied percentage proportion of each of the test solutions was poured into the labelled well (table 1) (Idris *et al.*, 2016)

Determination of the MIC of the assay samples BSE, HN & ZZ

A broth dilution method was used in order to determine the MIC of BSE, HN and ZZ on C. albicans. An over-night *C. albicans* culture was set-up in 5ml of SD broth. The fungal suspension was adjusted to 0.5 McFarland using SD broth. Doubled dilution of the samples (BSE, HN & ZZ) were prepared ranging from (60 mg/ml, 30mg/ml, 15 mg/ml, 7.5 mg/ml, 3.75 mg/ml and 1.88mg/ml) by diluting the stock sample in distilled water at 1:1 ratio. The MIC of the samples was determined using the broth dilution techniques. Twelve (12) sterile test tubes were placed in a test tube rack including 2 additional test tubes for control which contains SD broth. Using a pipette, 4ml of SD broth were dispensed into each of the 12 test tube. 0.1ml of the standardized inoculum was inoculated into each test tube using a sterile syringe and 0.6ml of the total treatment/samples were dispensed in the labeled tubes containing the SD broth and the fungal suspensions (see appendix; table 1). The treatments test-tubes were incubated at 37^0C for 24 hours. After an overnight stay, the tubes were examined for the presence of fungal growth which is usually express as turbid appearance of the tubes solutions. The dilution that shows no visible sign of growth (turbidity) was taken as the Minimum Inhibitory Concentration (MIC) (Idris *et al.*, 2016)

Determination of the MFC of BSE, HN & ZZ

After over-night incubation, the fungal suspensions from the test-tubes were sub-cultured on SDA plates and incubated at 37^0C for 24 hours. The concentration that shows no visible growth after the incubation was considered as the Minimum Fungicidal Concentration (MFC) (Silveira et al., 2009).

Demonstration of Synergetic Activity of the Extract, Honey and the Zam-zam water

Variable proportions of the concentrations were prepared and labeled as shown in the table below.

To obtain the exact volume of each treatment in the first well, we use the C1V1 = C2V2 equation to substitute our values.

Therefore, the vol of Black seed extract in the first treatment will be calculated as

C1 =?, C2 = 0.6ml (the total amount of treatment in each well), V1 = 80% and V2 =100%

C1 = 0.6 x 80/ 100 = 0.48 ml

For Honey,

C1 =?, C2 = 0.6ml, V1 = 10% and V2 = 100

C1 = 0.6 x 10/100 = 0.06ml

For Zam-zam,

C1 =?, C2 = 0.6ml, V1 = 10% and V2 = 100
C1 = 0.6 x 10/100 = 0.06ml
Thus, the total volume of 0.6ml is achieved by the summation of the above C1 Obtained
0.48 + 0.06 + 0.06 = 0.6ml.
The above mathematical expression is used therefore to obtain the exact volume/percentage of the test sample for each well/treatment used in this study.

RESULTS

Antifungal effect of BSE, HN & ZZ against *C. albicans*

Table 1: Result of the antifungal effect of BSE, HN & ZZ on *C. albicans*

	Zone of inhibition (mm)										
BSE (mg/ml)				**HN(mg/ml)**				**ZZ(mg/ml)**			
60	**30**	**15**	**7.5**	**60**	**30**	**15**	**7.5**	**60**	**30**	**15**	**7.5**
12	9	4	0	14	10	6	2	2	0	0	0

Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of BSE, HN & ZZ

Table 2: Result showing the MIC and MFC of BSE, HN & ZZ on *C. albicans*

Samples	MIC (mg/ml)	MFC (mg/ml)
Black Seed Extract	30	60
Honey	30	60
Zam-zam	NA	NA

Keys: NA= No activity

Synergistic Inhibitory Effect of the BSE, HN &ZZ against *C. albicans*

Table 3: Synergistic antifungal activity of BSE, HN & ZZ

Treatment/Well	**Zone of inhibition (mm)**
A	16
B	10
C	8
D	6
E	15
F	12
G	10
H	6
I	2
J	4
K	6
L	8
M	12
N	0

N = control

DISCUSSION

Previous and current researches about microbial resistance are focused more on bacteria than are on fungi. This is as a result of the wide-spread of bacterial infection, mostly nosocomial infections among humans and animal. In addition to this, resistance to therapeutic is mostly observed among bacterial organisms. However, the rate of resistance to antifungal agents is also a course for alarm, as fungal organisms that show resistance to antifungal can acquire secondary mechanisms in thwarting the effects of this therapeutics (Nathan, 2017). *Candida spp* such as *C. krusei* that causes hematological melignancies and *C. lusitaniae* that is responsible for candidemia are reported to be resistance to fluconazole and amphotericin B respectively (Lackner *et al.*, 2014). Worrisomely, many *Candida spp* that are attributed to infection among immunocompromised patients are reported to be resistance to a wide-range of antifungal which makes these organisms to be multi-drug resistance pathogens. *C. galbrata* and *C. auris* are urogenital and deadly blood stream pathogens that are reported to be resistance to both azole and echinocandin antimicrobials (Healey *et al.*, 2016).

In view of this, many investigations are currently carried out in order to attenuate antifungal resistance (Nathan, 2017). In this study, we observed that the synergistic activity of black seed extract, honey and zam-zam have some therapeutic effect on *C. albicans*.

The synergistic activity was observed to be highest at 80%, 10% and 10% (BSE, Honey and Zam-zam) treatment with 16mm zone of inhibition, followed by 10%, 80% and 10% (BSE, Honey and Zam-zam) treatment with 15mm. It has been reported by Khan *et al.*, in 2003 that the aqueous extract of *N. sativa* seeds exhibits inhibitory effect against *Candida* in mice. Al-Waili, 2001, also shows an in-vitro inhibitory effect of honey against *C. albicans*. Different extracts of *N. sativa* have a broad antimicrobial spectrum on different pathogenic bacteria such as *Salmonella typhi*, *Pseudomona aeruginosa* and *Bacillus aureus* (El-Hack et al., 2016). However, Zamzam water was found to be inactive against *C.albicans*.

In the attempt to assay the antifungal activity of the BSE, HN, and Zam-zam (table 1), we evaluate our findings in correspondence with the Clinical and Laboratory Standards Institute (CLSI) break-point for *C. albicans* MIC on certain drugs as demonstrated by Annett *et al.*, 2014 and CLSI, 2008. As the highest concentration of our test samples is 60mg/ml which is equivalent to 0.06µg/ml, we can deduced that BSE at 12mm, 9mm, and 4mm (60mg/ml, 30mg/ml and 15mg/ml), Honey (HN) at 14mm. 10mm, and 6mm (60mg/ml, 30mg/ml and 15mg/ml) are more effective on *C. albicans* than anidulafungin, micafungin and voriconazole synthetic therapeutics going by the revised CLSI MIC break-point on *C. albicans* (Annett *et al.*, 2014). In addition going by Annett et al., 2014, zamzam at 2mm has some antifungal effect on *C. albicans*.

CONCLUSION

Therefore, we concluded that BSE, and Honey have a great potency of inhibiting the growth of *C. albicans*. Although, our results have indicated a little activity of Zam-zam on *C. albicans*, however at 2mm, Zam-zam has the same break-point as some antifungals. We therefore, recommend a further comparative study on the effectiveness of BSE, honey and zam-zam with antifungals in order to prove this study further.

REFERENCES

Ahmad, A., Husain, A., Mujeeb, M., Khan, S.A., Najmi, A. K., Siddique, N. A., Damanhouri, Z. A., and Anwar, F. (2013): A review on therapeutic potential of *Nigella sativa*: A miracle herb. *Asian Pac J Trop Biomed.* **3**(5): 337–352

Al-Bukhari Division (71) on medicine: SahiAl-Bukhari The collection of authentic sayings of Prophet Mohammad (peace be upon him). 2nd edition 1976.

Al-Waili N. S (2001): Therapeutic and prophylactic effects of crude honey on chronic seborrheic dermatitis and dandruff. *Eur J Med Res*. **6**(7):306-8.

Al-Zuhair, N. and Khounganian, R. (2006): A comparative study between the chemical composition of potable water and Zam-zam water in Saudi Arabia. www.researchgate.net

Analytical report of Zamzam water cited from the annual report of the ministry of agriculture and water resources (1971).

Annette W. F, Deanna A. S, Dora I. M, and Nathan P. W. (2014): Impact of New Antifungal Breakpoints on Antifungal Resistance in Candida Species. *J Clin Microbiol*. **52**(3): 994–997

Arévalo, M. P., Carrillo-Muñoz, A. J., Salgado, J., Cardenes, D., Brió, S., Quindós, G. and Espinel-Ingroff, A. (2003): British Society for Antimicrobial Chemotherapy Antifungal activity of the echinocandin anidulafungin (VER002, LY-303366) against yeast pathogens: a comparative study with M27-A microdilution method. *Journal of Antimicrobial Chemotherapy* **51**, 163–166 DOI: 10.1093/jac/dkg018 163

Botanical Society of Britain and Ireland. Archived from the original (2015)

CLSI 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard—3rd ed. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA

CLSI (2014): Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard, 9th edition, CLSI document M07-A9. Clinical and laboratory standards institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

El-Hack, M. E. A., Alagawany, M., Farag, M. R., Tiwari, R., Karthik, K. and Dhama, K. (2006): Nutritional, Healthical and Therapeutic Efficacy of Black Cumin (*Nigella sativa*) in Animals, Poultry and Humans. *Inter. J. of Pharmaco*. **12** (3): 232-248

Erdogan A and Rao S. S (2015). "Small intestinal fungal overgrowth". Curr Gastroenterol Rep. 17 (4): 16. doi:10.1007/s11894-015-0436-2. PMID 25786900.

Healey K. R, Zhao Y, Perez W. B, Lockhart S. R, Sobel J. D, Farmakiotis D, Kontoyiannis D. P, Sanglard D, Taj-Aldeen S. J, Alexander B. D, Jimenez-Ortigosa C, Shor E, Perlin D.S (2016): Prevalent mutator genotype identified in fungal pathogen Candida glabrata promotes multi-drug resistance. Nat Commun, **7**:11128

Idris, U. A., Tijjani, M. B. and Aliyu, M. S. (2016): Evaluation of Synergetic Activity of Honey and Ginger Extracts on *Pseudomonas aeruginosa* and Methicillin-Resistant *Staphylococcus aureus*. *UMYU, J. Microbiol. Res.* **1**(1).

Khan, M. A, Chen, H., Tania. M., and Zhang, D. (2011): Anticancer Activities of *Nigella Sativa* (Black Cumin). *Afr J Tradit Complement Altern Med.* **8**(5 Suppl): 226–232

Khan, M. A., Ashfaq M. K, Zuberi, H. S., Mahmood, M. S., and A. H. Gilani, A. H. (2003): The in vivo antifungal activity of the aqueous extract from Nigella sativa seeds. *Phytotherapy Research*

Krishnapura Srinivasan (2018): Cumin (Cuminum cyminum) and black cumin (Nigella sativa) seeds: traditional uses, chemical constituents, and nutraceutical effects. *Food Quality and Safety*, 2(1), pp 1–16

Lackner M, Hagen F, Meis JF, Gerrits van den Ende AH, Vu D, Robert V, Fritz J, Moussa TA, de Hoog GS (2014): Susceptibility and diversity in the therapy-refractory genus scedosporium. *Antimicrob Agents Chemother*. **58**(10):5877-85.

Mandal, M. D. and Mandal, S. (2011): Honey: its medicinal property and antibacterial activity. *Asian Pac J Trop Biomed*. **1**(2): 154–160.

Nafiu, M. O., Hamid, A. A., Muritala, H. F. and Adeyemi, S. B. (2017): Medicinal spices and vegetables from Africa; Therapeutic potential against metabolic inflammatory, infections and systematic disease. 171-204.

Nathan P .W. (2017): Antifungal resistance: current trends and future strategies to combat. *Infect Drug Resist*.**10**: 249–259.

Salem M. L (2005): Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. *Int Immunopharmacol*. **5**(13-14):1749-70.

Silveira, C. P, Torres-Rodrı´guez, J. M, Alvarado-Ramı´rez, E., Murciano-Gonzalo, F., Maribel Dolande, Panizo, M. and Reviakina, V. (2009): MICs and minimum fungicidal concentrations of amphotericin B, itraconazole, posaconazole and terbinafine in Sporothrix schenckii. *Journal of Medical Microbiology*, **58**, 1607–1610

Subhash, C.P: Textbook of Practical Microbiology. 2006 edition.

Raju, S. B. and Rajappa, S. (2011): Isolation and Identification of *Candida* from the Oral Cavity. *ISRN Dent*. 487921. doi: 10.5402/2011/487921

Venkatachallam, S. K. T., Pattekhan, H., Divakar, S. and Kadimi, U. S. (2010): Chemical composition of *Nigella sativa* L. seed extracts obtained by supercritical carbon dioxide. *J Food Sci Technol*. **47**(6): 598-605.

Zam-zam Studies and Research Centre. Cited from The Saudi Geological Survey (2013).

Zarb, P. and Goossens, H. (2012); Human use of antimicrobial agents. Rev. Sci. Tech. **31**(1): 121-33.

Appendix 1

Table 1: Varied proportions used for the Synergistic activity of BSE, HN & ZZ on *C. albicans*

S/n.	Labeled Sample	% conc. of BSE	% conc. of HN	% conc. of ZZ	Vol. of BSE (ml)	Vol. of HN (ml)	Vol. of ZZ (ml)	Vol. of Distilled water (ml)
1	**A**	80	10	10	0.48	0.06	0.06	0.00
2	**B**	60	20	20	0.36	0.12	0.12	0.00
3	**C**	40	30	30	0.24	0.18	0.18	0.00
4	**D**	20	40	40	0.12	0.24	0.24	0.00
5	**E**	10	80	10	0.06	0.48	0.06	0.00
6	**F**	20	60	20	0.12	0.36	0.12	0.00
7	**G**	30	40	30	0.18	0.24	0.18	0.00
8	**H**	40	20	40	0.24	0.12	0.24	0.00
9	**I**	10	10	80	0.06	0.06	0.48	0.00
10	**J**	20	20	60	0.12	0.12	0.36	0.00
11	**K**	30	30	40	0.18	0.18	0.24	0.00
12	**L**	40	40	20	0.24	0.24	0.12	0.00
13	**M**	50	50	50	0.30	0.30	0.30	0.00
14	**N**	0.0	0.0	0.0	0.00	0.00	0.00	0.60