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Efficacy of Neem Kernel Bioactives Extracted using Supercritical Fluid Carbon Dioxide on Selected Dermatophytes and Foodborne Pathogens

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Abstract: ***Neem seed kernel was subjected to supercritical fluid carbon dioxide (SCF) process and evaluated for the inhibition of selected dermatophytes such as Candida albicans, Candida tropicalis, Candida parapsilosis, Trichophyton rubrum, Trichophyton mentagrophytes. The extraction was carried out by SCF-CO_2 at 300 bar 50 °C with and without entrainer (30 % methanol and acidified methanol). Results showed that the inhibition of the growth of all the dermatophytes is maximum in the extract obtained at 300 bar 50 °C with methanol (SCF-Me) followed by 300 bar 50 °C without methanol (SCF). The acidified SCF (SCF-MeA) extract did not show any remarkable inhibition. Of the dermatophytes, SCF-Me showed the inhibition against Candida parapsilosis of 18.46±0.25 mm compared to Candida albicans 16±0.26 mm, whereas the methanol control exhibited 10±0.3 mm inhibition. Out of the two fungal organisms, SCF-Me inhibited the growth of Trichophyton rubrum and Trichophyton mentagrophytes at 21.53±0.23 mm and 18.1±0.1 mm respectively. The extracts were also evaluated for its inhibition against the foodborne pathogens (bacteria, fungi, and yeast). The SCF-Me extract showed maximum inhibition against Pseudomonus aeroginosa (27.26±0.25) compared to Bacillus subtilis (24.36±0.35) at the concentration of 70mg. In fungus, Aspergillus ochraceus (16.7±0.60) was maximum when compared to Aspergillus niger (15.2±0.43) and yeast exhibited the inhibition of 15.93±0.20 at the concentration of 80mg. The dermatophytic and antimicrobial activity of the SCF-Me extract is due to the presence of higher amount of triterpenoinds such as Azadirachtin (50.7 %), 6-deacetyl nimbin (0.14 %), Nimbin (0.08 %), Salannin (1.83 %) and Epoxy azadiradione (1.58 %) which were confirmed by liquid chromatography and mass spectroscopy (LC-MS).***

Key words: ***Neem, Dermatophytes, Supercritical fluid carbon dioxide, high performance liquid chromatography.***

I. INTRODUCTION

The usage of Neem (*Azadirachta indica* A Juss) seed kernel and leaves have a long history of antibiotic properties such as antimicrobial, antifungal, antibacterial and antiviral for curing the diseases [1.2]. Dermatophytes are the major source of superficial mycoses of man and remain a public health problem especially in tropical and subtropical countries [3.4].

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Extraction of herbal plants by supercritical fluid carbon dioxide extraction method (SCF-CO_2) has received special attention to get biologically active products [5] due to the low viscosity and high diffusivity of carbon dioxide [6.7].Fractions obtained after extraction are rich in specific components near-natural form. Neem seed contains 40-45 % of oil and the remaining is a cellular matrix [8]. Limonoid class of triterpenoids [9.10.11] are found in neem oil such as azadirachtin (azadirachtin A), salannin, nimbin, nimbinin, nimbidin, 3-tigloylazadirachtol (azadirachtin B), and 1-tigloyl-3- acetyl-11-hydroxymeliacarpin (azadirachtin D). It has been reported [12] that the neem kernel oil contains antimicrobial property against a variety of pathogens. Dermatophytes cause dermatophytosis which is a superficial skin infection. The species of *Candida* and *Trichophyton* are majorly responsible for skin infection. Rebell *et al* (2001) [13] describe that the dermatophytes (*Trichophyton* species) and their substrates involved in tinea infection. The dry leaves of neem have been reported [14] to have the potential to colonize *candida albicans*. Natarajan *et al* (2002) [15] and the group suggests that the neem seed extracts are effective against *Trichophyton* infection.

Agricultural commodities and food feeds get contaminated by a group of Aflatoxins (AFs) produced by Aspergillus in favourable conditions of relative humidity and temperature. Different research has been carried out to inhibit the AFs contamination of food crops [16.17]. Razzaghi-Abyaneh *et al* (2005) [18] reported that aqueous extracts of neem leaf and seed can inhibit aflatoxin production in fungal mycelia. Neem leaf extracts considered to have a mixture of triterpenoids which produce excellent antifungal activity [19].

In view of these reports or results, it is understood that this infection is treated tropically and systematically. Although there is research work available on antidermatophytic as well as antimicrobial activity using conventional extracts but with SCF-CO_2 extracts is very scanty. The study was undertaken to employ SCF-CO_2 at different processing conditions and their effect on evaluating the antidermatophytic activity and antimicrobial activity against foodborne pathogens.

II. MATERIALS AND METHODS

A. Plant material

The neem seeds were locally procured from the localmarket of Mysore, Karnataka, India.

B. Chemicals

All the chemicals were procured from Merck, Mumbai. Terbinafine, Fluconozole, Tetracycline and Nystatin were obtained from pharmacy stores.

C. Microbial cultures and media

The bacterial and fungal strains viz., Candida albicans (ATCC 90028), Candida tropicalis (ATCC 750), Candida parapsilosis (ATCC 22019), Trichophyton rubrum (ATCC 28188), Trichophyton mentagrophytes (ATCC 9533), Bacillus subtilis (MTCC 8509), Bacillus cereus (MTCC 1305), Escherichia coli (MTCC 118), Pseudomonus aeroginosa (MTCC 4673), Aspergillus niger (MTCC 281), Aspergillus ochraceus (MTCC 4643), Saccharomyces cerevisiae (MTCC 173), procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. Laboratory requirements and media like nutrient agar (NA), nutrient broth (NB), potato dextros agar (PDA), potato dextros broth (PDB), Sabouraud dextrose agar (SDA) were purchased from Hi-Media Ltd., Mumbai, India.

D. Extraction of Neem kernel bioactives

Seeds were dried in cabinet tray drier for 24 h and dehulled by DIAF huller (Crompton Parkinson Ltd, Bombay). The kernel was separated and powdered in a hammer mill (CMC-CM; Cadmach Machinery Pvt.Ltd, Ahmedabad, India). Neem kernel powder (1 kg) was loaded to SCF-CO_2 extractor. Extraction was carried out at different pressure and temperature (100 bar / 40 °C, 150 bar / 50 °C, 200 bar / 40 °C and 200 bar / 50 °C) to remove volatiles. Further, the spent was extracted at 300 bar and 50 °C (SCF); 300 bar and 50 °C + 30 % methanol (SCF-Me); 300 bar and 50 °C with acidified methanol (SCF- MeA) to obtain the triterpenoids rich fractions.

The Conventional extraction of neem kernel powder was carried out in a soxhlet extractor. The powdered neem kernel (1 kg) was defatted with hexane and further extraction was done by methanol for six hour. The extract was concentrated using rota vapour at 40 °C. The yield (Table I) of extract was noted. Both SCF-CO_2 and conventional extracts were tested against dermatophytes and foodborne pathogens.

E. Microbial studies

F. Inoculums preparation of dermatophytes

The anti-candida activity of neem extracts was studied by agar well diffusion susceptibility test. All the species of Candida and Trichophyton used in this study were grown by using PDA medium. The cells were harvested and suspended in Tween 20 solution (0.1 % v/v). The number of cells was adjusted to 5 x 10^6 cfu mL-1 by using a hemocytometer. Each well was suspended with 50 µl of extract (60 mg, 80 mg) to identify the inhibition for Candida and *Trichophyton* species. All the plates were incubated at 30 °C for 24 h and 48 h. The plates were observed for the colonies and measured the inhibition zone.

G. Inoculum preparation of foodborne pathogens

The antibacterial and antifungal activity was studied against foodborne pathogens. Bacterial (Gram positive and Gram negative bacteria) and fungal cultures (*Apergillus niger, Aspergillus ochraceus and Saccharomyces cerevisiae*) were grown on NA and PDA medium, respectively. In Tween 20 solution (0.1 % v/v) the spores were suspended. On the surface of the medium, 1 ml of fungal suspension containing 10^6 cfu / mL was inoculated. The wells were bored and 50 µl of neem extracts, which includes the concentration of 70 mg for bacteria and 80 mg for fungal and yeast cultures were disposed. For 5 to 7 days, the plates were incubated and observed for mycelia growth for inhibition percentage.

H. Qualitative analysis (HPLC and MS)

The extracts were analyzed by HPLC (Shimadzu LC 10A, Japan) system, coupled with a photodiode array detector with a quaternary pump, working in the range of 190-810 nm. A reverse phase C_{18} column (15µ-Diamonsil, 250 mm x 4.6 mm) was used and the absorbance was monitored at 217 nm. Methanol (A) and water (B) were used as the mobile phase. The separations were achieved by a linear gradient of 40 % to 30 % B over 0-60 min at flow rate of 1.0 mL / min. Injection volume was 10 µl (10 µg).

Liquid chromatography and mass spectroscopy (LC-MS) analysis were performed by Water system 2695 separation module with auto sampler and photodiode array detector. The extract separation was done on a C-18 column (Phenomenex, Torrance, CA). The mobile phase contains methanol (A) and water (B), which were applied as the same HPLC gradient program. The mass spectra (MS) were obtained in positive electrospray mode with the ionization voltage of 70 V, source voltage of 3.5 kV. The mass scan range from 200 to 900 m/z and scan speed is 1000 amu/sec. Data processing and acquisition was done with the software Masslynx 4.0. Equilibration time 10 min was set after each run.

I. Statistical analysis

All the experiments were performed in triplicates. Data were analyzed for average mean standard deviation.

III. RESULTS AND DISCUSSION

The susceptibility of microorganisms to SCF-CO_2 as well as conventional extract were compared and also with selected positive control. A phytochemical constituent from neem seeds indicates that the natural products from plants widely used in the prevention and treating of skin and foodborne pathogen diseases [20.21].

A. SCF-CO_2 extraction of neem triterpenoids

Volatiles and less polar compounds (27.7%) were first removed at lower pressure by varying different conditions of temperature. Further, spent was subjected to three different experimental conditions. Extraction carried out at 300 bar and 50 °C with carbon dioxide alone yielded neem extract of 15 %. SCF-CO_2 experiment carried out with the same condition of pressure (300 bar) and temperature (50 °C) along with 30 % methanol and with acidified (0.1 N) methanol yielded the extracts of 14.15 % and 9 % respectively. The methanol extract of neem kernel obtained by soxhlet extraction is 7.2 % (Table I).

Table-I: Extraction yield of neem kernel by Supercritical fluid process and conventional extraction

Method of extraction	Conditions	Yield (g / kg)
SCF-CO_2	SCF	112.28
	SCF-Me	86.47
	SCF-MeA	48.51
Conventional	Soxhlet extraction	72.23

B. Characterization and identification of neem extracts

Chromatogram results of HPLC and LC-MS analysis show that the main components of neem extracts are Azadirachtin, 6-deacetyl nimbin, nimbin, salannin and epoxy azadiradion. SCF-CO_2 and conventional extracts chromatogram obtained by HPLC and LC-MS is comparable with result that reported in the literature [22.23]. Other trace amounts of triterpenoids exist are not identified in our study. The major components were identified on the basis of their relative retention time (RRT). The triterpenoid content was relatively higher (87 %) in 30 % methanol (entrainer) than without methanol (78 %) and with acidified methanol (69%). The highest yield is due to the polarity of each compound. Hence, the extraction yield is strongly dependent on the polarity of the extraction of the solvent. Carbon dioxide as a solvent has a limited ability to dissolve compounds of high-polarity. Therefore, the addition of entrainer to carbon dioxide can increase the solute solubility by increasing extraction efficiency.

The compounds were identified based on the molecular mass of individual compounds. The mass spectrum of Azadirachtin showed an [M+H] at m/z 721 protonated molecule, but sodium adduct ion [M+Na] + was relatively intensed at m/z 743. This suggests that it is caused due to the presence of traces of Na+ in the HPLC grade solvent. Due to the elimination of water [M+H-H_2O] + the base peak m/z 703 was formed. However, the salannin and nimbin molecules showed the mass spectrum as [M+H] + protonated molecule at m/z 597 and m/z 541 respectively, which is similar to the azadirachtin molecule also. The spectra of salannin and 6-desacetylnimbin (m/z 499) exhibit very strong [M+H] + ions and little fragmentation [23]. In the lower mass range, apart from common fragments epoxyazadiradione [24] showed a characteristic peak at m/z 467 ([M+H] +).

C. Quantification of compounds

The SCF-Me extract showed maximum Azadiractin content 507 µg / mg compared to all other extracts. This may be due to the solubility of azadirachtin in SCF-CO_2 along with the polar entrainer. As expected, the content of azadirachtin was least in SCF-MeA since azadirachtin was completely eluted with SCF-Me. The concentration of 6-deacetyl nimbin in all the extracts is in the range of 3 to 48 µg / mg. As seen from the table (Table II) the composition of nimbin (202 µg / mg) and salannin (209 µg / mg) is maximum at 300 bar 50 °C which indicates that these limonoids have the solubility in SCF-CO_2 even without the presence of entrainer. The result of the present study was similar to the result obtained by Ismadji et al (2013) [25]. The SCF-CO_2 acidified MeOH eluted 477µg / mg of epoxy azadiradione was comparatively more than without methanol (53 µg / mg) and with methanol (158 µg / mg). Which indicates this compound is eluted favourably in an acidic condition of the extracting solvent.

Azadiractin yield by the conventional method of extraction is 386 µg / mg, which is slightly lesser than SCF-Me. This is due to the solubility of azadirachtin in methanol with CO_2. The concentration of the other limonoids is 460 µg / mg, which constitutes 46 % of the total extract.

Table –II: HPLC analysis of yield of triterpenoids in the extracts

Compound	Retention time (RRT)	Actual Molecular Weight	Predicted Molecular Weight	SCF		SCF-Me		SCF-MeA		Conventional methanol extract	
				µg	%	µg	%	µg	%	µg	%
Azadirachtin	2.789	720	743	1.86	18.6	5.07	50.7	0.83	8.3	3.86	38.6
6-deacetyl nimbin	4.643	498	499	0.48	4.8	0.14	1.4	0.03	0.3	0.82	8.2
Nimbin	7.667	540	541	2.02	20.2	0.08	0.8	0.47	4.7	0.82	8.2
Salannin	17.55	596	596	2.95	29.5	1.83	18.3	0.87	8.7	0.41	4.1
Epoxy azadiradione	36.57	466	467	0.53	5.3	1.58	15.8	4.77	47.7	2.55	25.5

D. Anti-dermatophytic activity

The fungal strains were inhibited by extracts of both SCF-CO_2 and conventional extracts of neem. However, neem extract obtained by SCF-Me showed more significant antidermatophytic activity compared to SCF, SCF- MeA and conventional extracts (Table III). The neem seed kernel extract of SCF-Me exhibited antifungal activity against *Candida* species. Maximum inhibition (18.46 ± 0.25 mm inhibition zone) was found in the case of *C. parapsilosis* followed by *C. albicans* with 16 ± 0.26 mm and *C. tropical* is 13.93 ± 0.90, respectively. Neem seed kernel SCF- MeA exhibited medium inhibition with a maximum of 15.36 ± 0.47 mm for *C. parapsilosis* followed by *C. tropicalis* (12.33 ± 0.41 mm) and *C. albicans* (12 ± 0.3 mm). The neem seed extract of SCF did not show any inhibition for *C. tropicalis.* But in *C. parapsilosis* and *C. albicans* inhibition was recorded was 14.3 ± 0.36 mm and 13 ± 0.3 mm, respectively. The extract concentration was needed to inhibit the microorganisms was 60 mg / mL. Charmaine *et al* (2005) [26] studied the inhibition activity against extracts of seed kernel of neem on *Candida* species isolated from patients infected with HIV. It is reported that neem extract of ethanol and hexane, concentration ranging from 1 to 0.0625 mg / mL showed best results.

Table –III: Inhibition of Supercritical fluid extracts of Neem kernel against dermatophytes

Extracts	Concentration (mg)	*Candida albicuns*	*Candida tropicalis*	*Candida parapsilosis*	Concentration (mg)	*Trichophyton rubrum*	*Trichophyton mentagrophytes*
SCF	60	13 ± 0.3	-	14.3 ± 0.36	80	18.03 ± 0.45	16.33 ± 0.41
SCF-Me	60	16 ± 0.26	13.93 ± 0.90	18.46 ± 0.25	80	21.53 ± 0.23	18.1 ± 0.1
SCF-MeA	60	12 ± 0.3	12.33 ± 0.41	15.36 ± 0.47	80	14.06 ± 0.40	13.33 ± 0.35
Conventional	60	-	-	12.23 ± 0.32	80	10.13 ± 0.23	11.1 ± 0.55
Methanol (control)	50 µl	10 ± 0.30	10 ± 0.41	-	50 µl	10 ± 0.23	10 ± 0.05
Terbinafine	12.5	26.5 ± 0.62	33.2 ± 0.34	36.03 ± 0.05	12.5	29.5 ± 0.45	26.06 ± 0.11
Fluconozole	7.5	25.2 ± 0.4	27.9 ± 0.50	37.66 ± 1.06	7.5	26.9 ± 0.15	23.16 ± 0.28

Similar trends of inhibition were observed in *Trichophyton* (Table III) species. SCF-Me neem seed kernel extract showed maximum inhibition (21.53 ± 0.23 mm) against *Trichophyton rubrum* followed by *Trichophyton mentagrophytes* (18.1 ± 0.1). SCF-MeA neem seed kernel extract exhibited less inhibition 14.06 ± 0.40 mm (*T. rubrum*) and (*T. mentagrophytes*) 13.33 ± 0.35 mm, respectively. SCF neem kernel extract was recorded to show inhibition of 18.03 ± 0.45 mm against *T. rubrum and* 16.33 ± 0.41 mm *T. mentagrophytes.* The concentration of all the extracts, which is used to inhibit *Trichophyton* microorganism is 80 mg / mL.

Natarajan *et al* (2003) [4] determine the activity of neem seeds and leaves on clinical isolates of dermatophytes and reported that the minimum inhibitory concentration (MIC) of neem seed extracts was 31 µg / mL for all dermatophytes and 15 µg / mL concentrations below MIC was sufficient for distorting the growth pattern of the dermatophytes. These results suggest that it is due to the presence of secondary metabolites (triterpenoids).

Results show that the antidermatophytic activity may be due to the presence of chemical constituents such as Azadirachtin, 6-deacetyl nimbin, nimbin, salannin and epoxy azadiradion. Similar results are obtained by Dube *et al* (1987) [27] who showed that the neem aqueous extracts obtained from leaf and bark, inhibited spore germination as well as mycelial growth of *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Microsporum canis*.

Among the foodborne pathogens tested with extracts of neem SCF-CO_2 and conventional extracts, maximum inhibition (Table IV) was exhibited SCF-Me extract. At a concentration of 70 mg / ml, highest inhibition (27.26 ± 0.25 mm) was found against *Pseudomonus aeroginosa* and 24.36 ± 0.35 mm for *Bacillus subtilis*. In fungal and yeast culture (Table V), 15.2 ± 0.43 mm (*Aspergillus niger*), 16.7 ± 0.60 mm (*Aspergillus ochraceus*) and 15.93 ± 0.20 mm showed the inhibition. Neem SCF-CO_2 with acidified methanol exhibited the minimum inhibition. Likewise, Grewal *et al* (1988b) [28] reported that neem seed aqueous extract inhibited the growth of 11 out of 21 test fungi. However, the drawback of this study is that the concentration of extract which is used in this study is higher and there will be chances of cross contamination when extracted with the aqueous medium. This drawback can overcome when we extract with SCF-CO_2 because extraction will be done in an isolated system.

Govindachari *et al* (1998) [20] who found that a mixture of neem fractions eluted from HPLC was recorded more effective when assayed against antifungal activity when compared to purified individual compounds. This shows that triterpenoids when purified separately, it loses effect, whereas in the mixture it gives the excellent antifungal activity. This proves that an additive synergism occurs among them.

E. Antimicrobial activity

Table IV. Effect of Supercritical fluid extracts of Neem on foodborne bacterial organisms

Extract	Concentration (mg)	Gram positive bacteria		Gram negative bacteria	
		Bacillus subtilis	*Bacillus cereus*	*E-coli*	*Pseudomonus aeroginosa*
SCF	70	19.26 ± 0.25	17.06 ± 0.11	22.3 ± 0.26	25.43 ± 0.45
SCF-Me	70	24.36 ± 0.35	21.36 ± 0.35	26.26 ± 0.45	27.26 ± 0.25
SCF-MeA	70	16.23 ± 0.40	14.46 ± 0.25	18.53 ± 0.41	17.5 ± 0.45
Neem Soxtech	70	12.23 ± 0.25	-	11.03 ± 0.45	-
Methanol (control)	50 µl	10 ± 0.41	10 ± 0.30	10 ± 0.05	10 ± 0.23
Tetracycline	10 µl / mL	28.93 ± 0.20	26.3 ± 0.26	28.46 ± 0.50	26.13 ± 0.15

Table V. Effect of Supercritical fluid extracts of Neem on food borne yeast and fungal organisms

Extract	Concentration (mg)	Fungus		*Yeast*
		Aspergillus niger	*Aspergillus ochracieus*	*Saccharomyces cerevisiae*
SCF	80	12.96 ± 0.25	15.26 ± 0.30	15.1 ± 0.36
SCF-Me	80	15.2 ± 0.43	16.7 ± 0.60	15.93 ± 0.20
SCF-MeA	80	11.53 ± 0.45	12 ± 0.7	12.33 ± 0.30
Conventional	80	10.06 ± 0.66	10.83 ± 0.47	10.26 ± 0.45
Methanol (control)	50 µl	6.5 ± 0.25	6.5 ± 0.28	6.5 ± 0.05
Nystatin	2 µl / mL	28.93 ± 0.30	25.9 ± 0.30	32.16 ± 0.55

IV. CONCLUSIONS

In conclusion, the microbial activity of SCF-Me neem extract was considered to be more effective when compared with SCF, SCF-MeA and conventional fractions. SCF-Me showed the maximum inhibition against *Candida parapsilosis* (18.46 ± 0.25 mm) and *Trichophyton rubrum* (21.53 ± 0.25 mm) at a concentration of 60 and 80 mg respectively. Gram negative bacteria was more sensitive to SCF-Me fraction when compared to gram positive bacteria, fungi and yeast. Inhibition of microorganism's growth may be due to the presence triterpenoids in precise concentration and also due to the synergetic action of the compounds with cellular and membrane components.

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REFERENCES

1. Ezeonu, C.S., Imo, C., Agwaranze, D.I., Iruka, A. and Joseph, A. 2018. Antifungal effect of aqueous and ethanolic extracts of neem leaves, stem bark and seeds on fungal rot diseases of yam and cocoyam. Chemical and Biological Technologies in Agriculture. 5:18. https://doi.org/10.1186/s40538-018-0130-3.
2. Susmitha, S., Vidyamol, K.K., Ranganayaki, P. and Vijayaragavan, R. 2013. Phytochemical extraction and antimicrobial properties of Azadirachta indica (Neem). Global Journal of Pharmacology, **7**, 316–320.
3. Venugopal, P.V. and Venugopal, T.V. 1993. Antimycotic susceptibility testing of dermatophytes. Indian Journal of Medical Microbiology,**11**, 151-154.
4. Natarajan, V., Venugopal, P.V. and Menon, T, 2003. Effect of *Azadirachta indica* (neem) on the growth pattern of dermatophytes. Indian Journal of Medical Microbiology, **21**, 98-101.
5. Danielski, L. 2007. "Extraction and fractionation of natural organic compounds from plant materials with supercritical carbon dioxide." Phd thesis, Technische University Hamburg-Harburg, Hamburg, pp. 181.
6. Canela, A. P. R. F., Rosa, P. T. V., Marques, M.O.M. and Meirelesm M.A.M, 2002. "Supercritical Fluid Extraction of Fatty Acids and Carotenoids from the Microalgae Spirulina maxima." Industrial & engineering chemistry research, **41**, 3012-3018.
7. Roy, B. C., Goto, M. and Hirose, T., 1996. "Extraction of ginger oil with supercritical carbon dioxide: experiments and modeling". Industrial and engineering chemistry research, **35**, 607-612.
8. Johnson, S. and Morgan, E.D., 1997. "Supercritical fluid extraction of oil and triterpenoids from neem seeds." Phytochemical Analysis, **8**, 228-232.
9. Mongkholkhajornsilp, D., Douglas, S., Douglas, P.L., Elkamel, A., Teppaitoon, W. and Pongamphai, S., 2005. Supercritical CO_2 extraction of nimbin from neem seeds - a modelling study, Journal of Food Engineering, 7**1**, 331-340.
10. Morgan, E. D., 2009."Azadirachtin, a scientific gold mine." Bioorganic & medicinal chemistry**, 17**, 4096-4105.
11. Melwita, E. and Ju Y.-H., 2010. "Separation of azadirachtin and other limonoids from crude neem oil via solvent precipitation." Separation and Purification Technology, **74**, 219-224.
12. Rao, D.V.K., Singh, K., Chopra, P., Chabra, P.C. and Ramanujalu, G., 1986. *In vitro* antibactericidal activity of neem oil. Indian Journal of Medical Research, **84**, 314-316.
13. Rebell, G. and Zaias, N., 2001. Introducing the syndromes of human dermatophytosis. Cutis, **67**, 6-17.
14. Polaquini, SR , Svidzinski, TI , Kemmelmeier, C and Gasparetto, A., 2006. Effect of aqueous extract from Neem (Azadirachta indica A. Juss) on hydrophobicity, biofilm formation and adhesion in composite resin by Candida albicans. Archives of Oral Biology, **51**, 482–490.
15. Natarajan, V., Pushkala, S., Karuppiah, V.P. and Prasad, P.V., 2002. Anti dermatophytic activity of Azardirachta indica (neem) by invitro study. Indian Journal of Pathology and Microbiology, **45**, 311-313.
16. Cleveland, T. E. and Bhatnagar, D., 1992. Molecular strategies for reducing aflatoxin levels in crops before harvest. In D. Bhatnagar and T. E. Cleveland (Eds.), Molecular approaches to improving food quality and safety. pp 205–228. New York: Van Nostrand Reinhold.
17. Philips, T. D., Clement, B. A., and Park, D. L., 1994. Approaches to reduction of aflatoxins in foods and feeds. In D. L. Eaton & J. D. Groopman (Eds.), The toxicology of aflatoxins: Human health, veterinary and agricultural significance, pp. 383–406. New York: Academic Press.
18. Razzaghi-Abyaneh, M., Allameh, A., Tiraihi, T., Shams-Ghahfarokhi, M. and Ghorbanian, M., 2005. Morphological alterations in toxigenic Aspergillus parasiticus exposed to neem (Azadirachtaindica) leaf and seed aqueous extracts. Mycopathologia, **159**, 565–570.
19. Mahmoud, D.A., Hassanein, N.M., Youssef, K.A. and Zeid, M.A.A., 2011. Antifungal activity of different neem leaf extracts and the nimonol against some important human pathogens. Brazilian Journal of Microbiology, **42**, 1007-1016.
20. Govindachari, T.R., Suresh, G., Gopalakrishnan, G., Banumathy, B. and Masilamani, S., 1998. Identification of antifungal compounds from the seed oil of Azadirachta indica. Phytoparasitica, **26**, 1-8.
21. Hoque, M.M.D., Bari, M.L., Inatsu, Y., Juneja, V.K. and Kawamoto, S., 2007. Antibacterial activity of guava (Psidiumguajava L.) and neem (A. indica A. Juss.) extracts against foodborne pathogens and spoilage bacteria. Foodborne Pathogens and Disease, **4**, 481-488.
22. Ambrosino, P., Fresa, R., Fogliano, V., Monti, S.M. and Ritieni, A., 1999. Extraction of azadirachtin A from neem seed kernels by supercritical fluid and its evaluation by HPLC and LC/MS, Journal of Agricultural and Food Chemistry, **47**, 5252–5256.
23. Schaaf O., Jarvis A. P., Andrew van der Esch S., Giagnacovo G. and Oldham N. J., 2000. Rapid and sensitive analysis of azadirachtin and related triterpenoids from neem from HPLC-atmospheric pressure chemical ionisation mass spectrometry. Journal of chromatography A, **886**. PP. 89-97.
24. Haldar, S, Phapale, P.B., Kolet, S.P. and Thulasiram, H.V., 2013. Expedient preparative isolation, quantification and characterization of Neem limonoids from Neem Fruits. Analytical Methods, **5**, 5386-539.

25. Ismadji, A., Kurniawan, Y.H., Ju, F.E., Soetaredjo, A. and Ayucitra Ong, L.K., 2013. Solubility of azadirachtin and several triterpenoid compounds extracted from neem seed kernel in supercritical CO_2, Fluid Phase Equilibria, **336**, 9-15.
26. Charmaine Lloyd, A.C., Menon, T. and Umamaheshwari, K., 2005. Anticandidal activity of Azadirachta indica. Indian Journal Pharmacology, **37**, 386-389.
27. Dube, S. and Tripathi, S., 1987. Toxicity of some plants against dermatophytes. National Academy of Sciences, India, Science Letters, **10**, 45-48.
28. Grewal, P.S. and Grewal, S.K., 1988b. Selective fungicidal properties of some plant extracts to mushroom weed moulds. Phytopathologia Mediterranea, **27**, 112-114. http://www.jstor.org/stable/42684907.

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