Safety assessment and evaluation of the antimicrobial activity of *Ochna obtusata* leaves extracts against food borne pathogens

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ABSTRACT

Natural preservatives have been playing an important role to make sure safe usage of freshly chopped vegetables and fruits. To ensure the antimicrobial activity on food pathogens, *Ochna obtusata* extracts were evaluated against Gram positive bacteria (*Staphylococcus aureus),* Gram Negative Bacteria *(Escherichia coli),* and two types of fungi *(Aspergillus niger,* and *Candida albicans).* The percentage yields of solvent extracts of different solvents obtained were calculated and the ethanolic extract turned out to show the highest extraction yield followed by ethanol, petroleum, and water. According to the agar disc diffusion study, the ethanolic extract had shown the maximum antibacterial activity. The minimum inhibitory concentrations (MIC's) was 121 for *Aspergillus niger*. Minimum concentrations of the extract as a bactericidal and fungicidal were determined for all the extracts and it was obtained that only ethanolic extracts exhibited antimicrobial activity. Few of the remaining solvent extracts though exhibited some antimicrobial activity the results were not uniform and hence could not justify themselves as a potent antimicrobial agent. It was therefore concluded that ethanolic extract of *Ochna obtusata* can be a potent alternative as a natural preservative against food-borne pathogens.

Keywords : Food Pathogens, *Ochna obtusata*, Antimicrobial, Bacteria, Fungi, Preservatives

Introduction:

Plants as an alternate source of medicine and primarily as a source of food has been a requirement since the existence of humankind. Many pieces of evidence report the traditional usage of several medicinal herbs and their parts for treating several problems in humans. With the increase in the treatment using synthetic drugs the hunt for medicinal plants has increased in the recent past (Knight, 2007). India has reportedly 11 plants belonging to the *Ochna* genus which are used in the treatment of epilepsy, menstrual complaints, lumbago, asthma, ulcers, and as an antidote to snake bites (Kirtikar, Basu and Blatter, 1980). 111 different chemical constituents were reported in the *Ochna* species and all reported as Flavonoids, Anthranoids, triterpenes, Steroids, Fatty Acids, and many other compounds. Out of these 43 compounds were Monoflavonoids, 43 were bioflavonoids, 2 were Triflavonoids, 1 was a Pentaflavonoid, 8 were anthranoids, 1 was a triterpene, 3 were steroids, 8 were fatty acids, 1 glucose derivative, and lastly a cyanoglucoside (Bandi *et al.*, 2012).

Ochna obtusata also known as the Mickey Mouse plant is known to have various traditional uses in India especially by the tribal community of Orissa. Various studies report the anti diabetic(Indukuri *et al.*, 2014), anti diuretic (B.Venkateswarlu; and Y.Rama, 2013), Antioxidant (V Ravi Kumar*, T Shivaraj Gouda, Sreelakshmi S, 2014), Anti-epileptic (Nagarajan *et al.*, 2013), Anti Inflammatory (V Ravi Kumar*, 2018), Anti-Ulcer (Karimulla and Kumar, 2012) and Hepatoprotective activity (Vakkalagadda and Lankalapalli, 2020) of *Ochna obtusata*. This is due to the reported pieces of evidence of the plant extract containing several phytoconstituents rich in Flavonoids, Phenolics, and terpenoids. Flavonoids due to their capability of attacking multiple cellular targets including cytoplasmic membrane functions, microbial metabolism, and microbial nucleic acid formation it has been reported to prove their antimicrobial activity.

Antibiotic resistance by food-borne microbes is bound to reduce their dependence on synthetic antimicrobial agents. The current research was designed to assess the effect of various extracts of *Ochna obtusata* against food-borne different pathogenic microbes.

Materials and Methods:

Collection and preparation of samples:

The leaves of *Ochna obtusata* were obtained from the forest of Tirumala hills and authenticated by Dr. Madhava Shetty, taxonomist, Department of Botany, Sri Venkateswara University; Tirupati, Andhra Pradesh. The collected leaves of *O.obtusata* were washed before chopping them into small pieces, and dried using a hot air oven in the laboratory until dry at low temperature to avoid

charring(Purkait *et al.*, 2018). It was then ground by using a mechanical grinder and sieved to obtain a fine powder to pass through 100mm sieve.

Preparation of *Ochna obtusata* extract*:*

Successive extraction of 100 gms of finely powdered leaves of *O.obtusata* was extracted using ethanol, petroleum ether, chloroform, and water using a dry weight to the solute-solvent ratio of 1:4 (v:w) for 7 days at room temperature. The flask was covered with aluminum foil to prevent the darkening of the extract. On the 8th day, the plant materials were filtered using a Whatmann filter paper No. 2. All the extracts were evaporated to dryness under vacuum using a rotary evaporator at 50^{O}C at a speed of 150 revolutions per minute to obtain a complete dry extract. The crude drug was standardized to 10mg/ml, 100mg/ml, 250mg/ml and 500mg/ml of stock extracts and stored at 4^{O}C until further usage.

Yield calculation:

The percentage yield of crude plant extracts was calculated on a dry weight basis using the formula:

$$\textbf{Yield of extracts}\ (\%) = \frac{\textbf{Weight of dry extract obtained}\ (\mathbf{g})}{\textbf{Weight of Crude Powder used for extraction}\ (\mathbf{g})})/* \mathbf{100}$$

Preparation of inoculums for antimicrobial activity:

Muller Hinton agar (MHA, Himedia, India), Sabouraud Dextrose Agar (SDA, Himedia India), and Potato Dextrose Agar (PDA, Himedia India) were used for the study. Four bacteria's were used in the study *Bacillus cereus, Staphylococcus aureus (Gram positive), Escherichia coli and Pseudomonas aeruginosa (Gram negative), whereas the fungi used were A.niger, and C.albicans.* The bacteria's were maintained in MHA whereas *A.niger* and *C.albicans* were maintained on PDA and SDA respectively (Wong and Ramli, 2021). Before starting the study, a single colony from bacterias pure cultures were transferred to Muller Hinton Broth (MHB; Himedia, India) and *A.niger* and *C.albicans* were transferred to PDB and SDB for fungi cultures respectively using a sterile inoculating loop.

Bacteria were cultivated for 24 hours at 37^{O}C on MHA, and *C.albicans* were propagated for 48 hours at 35^{O}C on SDA. A single colony of each bacteria and fungus was transferred from the media plate to MHB and SDB, and incubated at 37°C for 48 hours and 35°C for 48 hours, respectively, with 200rpm agitation. 1ml of overnight bacteria or fungal suspension in MHB or SDB was centrifuged for 1 minute at 3000 rpm or 3900 rpm, respectively. The pellet was retrieved and rinsed

twice in sterile water containing 0.85 percent sodium chloride. To standardise the McFarland turbidity of 0.5 (equivalent to 106 CFU / ml) for bacteria and fungi, sterile water was added.

For standardisation of *A.niger*, spores were collected by distributing 1 ml of sterile saline water (0.85 percent) onto 7-day old fungal colonies and covering them with it at 35^{O}C. To separate the particles, the spores were distributed into a 1.5 mL microcentrifuge tube. The heavy particles were allowed to settle before being collected and transferred to another sterile microcentrifuge tube (1.5 mL). Spore suspension density was diluted to an optical density of 80 to 82 percent transmittance using sterile water. The obtained suspension was diluted in sterile distilled water at a ratio of 1:50, resulting in approximately 104 CFU/ml.

Agar disc diffusion test:

The Agar disk diffusion method was used to study the susceptibility of microbial strains to plant extracts (Uwizeyimana *et al.,* 2020). The freshly prepared culture was spread on a fresh plate using a sterile cotton swab dipped in fresh prepared bacterial culture. The plates were dried in the laminar air flow hood for five minutes to absorb the excess moisture till dry, up to ten minutes. Sterile filter paper disc (6mm) was placed onto the surface of the agar plates using sterile forceps. 10 µL of different concentrations of *O.obtusata* extract (10, 100, 250, and 500 mg/ml) was placed on the filter paper. Chlorhexidine and Amphotericin were used as positive controls for bacteria and fungi respectively. All the plates were incubated at 37^{O}C for 24 hours (MHA) and 35^{O}C (PDA and SDA). Occurrence for any zones of inhibition was observed and measured and tabulated. MIC, MBC, and MFC tests were performed in triplicate for the concentration of extracts that exhibited effective inhibition.

Determination of MIC, MBC, and MFC

96 well sterile microtitre plate was used to evaluate the MIC for the extracts of *O.obtusata* against different tested strains with an inoculum of approximately 10^6 CFU/mL for bacteria and *C.albicans* and 10^4 CFU/mL for *A.niger*. Pure microbial culture without any antimicrobial culture was used as the positive control and pure medium without microbes was used as the negative control. The plates were incubated aerobically at 37^{O}C for 24 hours (MHA) and 35^{O}C (PDA and SDA). The lowest concentration that was able to inhibit the growth was recorded as MIC. 10 µL of suspension from each well from the MIC test was spotted on the appropriate media and incubated at 37^{O}C for 24 hours bacteria and 35^{O}C for fungi to determine the MBC and MFC (Ben Lagha *et al.*, 2019).

Statistical Analysis

The results of the experiment were expressed as Mean Standard Deviation. Using Microsoft Excel 2017 and SPSS, the numbers were tabulated and a standard calibrations curve was created. At a significance threshold of =0.05, a one-way ANOVA was utilised to find significant differences (P0.05) between different sample types.

Results:

Extraction yields of different extracts:

The percentage yield of ethanol chloroform petroleum ether and water extracts were found to be 14.8, 11.4, 7.4 and 5.8 % w/w respectively.

Antimicrobial activities:

The extracts were screened for its antimicrobial activity using the agar disc diffusion technique. Zone of inhibition was exhibited by ethanolic, chloroform and petroleum ether extracts as per the previous studies (Thobias M Kalenga *et al.*, 2021) whereas aqueous extract did not exhibit acceptable results. The antimicrobial study was performed for all the extracts at concentrations of 10mg/ml, 100mg/ml, 250mg/ml and 500mg/ml against the microorganisms. It was observed from **Table 1** that the ethanolic extracts possessed significantly higher zones of inhibition as compared to the other extracts thereby indicating itself to be the best among the different solvent extracts. It was found that only ethanolic extracts exhibited antimicrobial activity since it was found to produce zone of inhibitions in bacteria. However, it was found that no antifungal activity was exhibited by the ethanolic extracts at 10 mg/ml. It was also observed that chloroform and petroleum ether extracts at 100 mg/ml were found to be ineffective against the microorganisms be it bacteria's or fungi. The inhibition zones of Gram-Positive bacteria's were found to be on the higher end compared to the Gram-Negative bacteria's and Fungi used for the study. All the inhibition results were compared to the inhibition produced by standard antimicrobials (CHX and AMP B) used for the study (P<0.05; **Table 1**Error! Reference source not found.**).**

Table 1: Inhibition zones obtained for *Ochna obtusata* extracts against few food borne pathogens

Microorganisms	Zone of Inhibitions (mm)			Chlorhexedine / Amphoterecin B	Aqueous extract
	Ethanolic extract	Chloroform extract	Pet ether extract		
1% extracts (10 mg / ml)					

Bacillus cereus	7.09 ± 0.42	X	X	9.46 ± 0.001	X
Staphylococcus aureus	6.76 ± 0.78	X	X	8.82 ± 0.004	X
Pseudomonas aeruginosa	6.76 ± 0.78	X	X	8.98 ± 0.002	X
Escherichia coli	6.98 ± 0.56	X	X	8.95 ± 0.004	X
Aspergillus niger	X	X	X	8.12 ± 0.012	X
Candida albicans	X	X	X	8.02 ± 0.014	X
10% extracts (100 mg / ml)					
Bacillus cereus	6.47 ± 0.67	X	X	8.98 ± 0.002	X
Staphylococcus aureus	6.81 ± 0.45	X	X	8.98 ± 0.004	X
Pseudomonas aeruginosa	6.72 ± 0.34	X	X	8.95 ± 0.012	X
Escherichia coli	6.82 ± 0.56	X	X	8.82 ± 0.014	X
Aspergillus niger	X	X	X	8.12 ± 0.006	X
Candida albicans	6.89 ± 0.66	X	X	8.02 ± 0.004	X
25% extracts (250 mg / ml)					
Bacillus cereus	8.45 ± 0.34	6.89 ± 0.68	6.45 ± 0.32	8.44 ± 0.012	X
Staphylococcus aureus	8.57 ± 0.32	6.12 ± 0.35	6.59 ± 0.12	8.98 ± 0.006	X
Pseudomonas aeruginosa	8.56 ± 0.68	6.43 ± 0.56	7.08 ± 0.72	8.78 ± 0.003	X
Escherichia coli	8.62 ± 0.87	6.67 ± 0.32	7.25 ± 0.12	8.95 ± 0.004	X
Aspergillus niger	6.45 ± 0.87	6.01 ± 0.63	6.78 ± 0.89	8.92 ± 0.006	X
Candida albicans	7.82 ± 0.67	6.67 ± 0.34	7.01 ± 0.67	8.92 ± 0.002	X
50% extracts (500 mg / ml)					
Bacillus cereus	11.78 ± 0.64	9.81 ± 0.08	7.32 ± 0.36	8.46 ± 0.003	
Staphylococcus aureus	12.05 ± 0.45	9.86 ± 0.12	6.67 ± 0.46	8.98 ± 0.002	X
Pseudomonas aeruginosa	11.28 ± 0.45	9.12 ± 0.78	6.32 ± 0.39	7.12 ± 0.022	X
Escherichia coli	11.45 ± 0.67	8.56 ± 0.32	8.12 ± 0.32	8.95 ± 0.010	X
Aspergillus niger	12.89 ± 0.56	9.89 ± 0.38	7.04 ± 0.89	8.92 ± 0.003	X
Candida albicans	12.78 ± 0.46	10.49 ± 0.56	7.22 ± 0.45	8.88 ± 0.008	X

Based on the above findings the MIC's, the MBC's and MFC's were determined for the 250mg/ml and 500mg/ml of *Ochna obtusata* extracts. The other concentrations viz. 10mg/ml and 100mg/ml were not considered because except for the ethanolic extracts they were found to be ineffective as per the study. The MIC's and MBC's for *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli* were found to be 47.2 mg/ml, 68.9mg/ml, 26.8 mg/ml and 21.7 mg/ml whereas the MIC's and MFC's for *A.niger* and *C.albicans* were found to be 121 mg/ml and 11.25 mg/ml as indicated in **Table 2(a)** and **Table 2(b).**

Table 2(a): MIC, MBC, AND MFC of *Ochna obtusata* extracts (25%) against few food borne pathogens

Strains	Ethanolic Extract		Chloroform extract		Petroleum Ether extract	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
25% extract (250 mg / ml)						
Bacillus cereus	76.4	76.4	X	X	X	X
Staphylococcus aureus	79.5	79.5	X	X	X	X
Pseudomonas aeruginosa	71.6	72.2	X	X	X	X
Escherichia coli	62.5	62.5	X	X	X	X

Aspergillus niger	X	X	X	X	X	X
Candida albicans	121	121	X	X	X	X

Table 2(b): MIC, MBC, AND MFC of *Ochna obtusata* extracts (50%) against few food borne pathogens

Strains	Ethanolic Extract		Chloroform extract		Petroleum Ether extract	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
50% extract (500 mg / ml)						
Bacillus cereus	49.1	48.6	X	X	X	X
Staphylococcus aureus	47.2	47.2	X	X	X	X
Pseudomonas aeruginosa	29.5	28.4	X	X	X	X
Escherichia coli	26.8	26.8	X	X	X	X
Aspergillus niger	121	121	X	X	X	X
Candida albicans	11.25	11.25	X	X	X	X

Discussion:

The study is purposeful on evaluating the antimicrobial activity of the chosen solvent that must have the capability of extracting the antimicrobial components from the crude extract and the concentration may or may not be correlated with the extraction yield. Hence all the extracts were evaluated for their antimicrobial activities. Previous studies report the presence of phytochemicals like phenolics, flavonoids, and terpenoids in the ethanolic extracts which may have contributed to the antimicrobial activity of the plant extract (Vakkalagadda and Lankalapalli, 2020). The compounds may cause leakage of cellular components due to membrane disruption.

From the study, it was found that Gram-negative bacteria's were more susceptible towards the extracts which maybe because of its nature of having an extra outer membrane around itself thereby limiting the permeability and preventing itself from penetrating the cell wall (Tavares *et al.*, 2020) (Schwechheimer and Kuehn, 2015). From the study, it was also observed that *A.niger* could sustain the high doses of the plant extract and thereby concluding that the inhibition activity was weak in the case of *A.niger*. Previous studies indicate the limited data exist regarding the anti-fungal agents when compared to the antibacterial agents. It was also reported that *Aspergillus* species including *A.niger* exhibited low susceptibility towards antifungal agents with few examples such as amphoterecin B, azole, itraconazole. Posaconazole, caspofungin and voriconazole (Van Der Linden, Warris and Verweij, 2011) (Wu *et al.*, 2020) (Hashimoto *et al.*, 2017).

It has been reported that the effectiveness of plants extracts as natural preservatives in edible food is limited and also depends on the various laboratory systems, food matrices (Ogunnupebi *et al.*, 2020), and behavioural characteristics of microorganisms(Pabón-Baquero *et al.*, 2018). Also, *O.obtusata* has no evident reports of acting as a natural preservative. Hence, the study was performed for various concentrations to determine the effectiveness of the extracts on food

pathogens. From the study, it was therefore concluded that the antimicrobial susceptibility depends on the type of solvents used and the microbial strains tested. Ethanolic extract was found to be effective in extracting the bioactive compounds present in *O.obtusata*. Hence further studies may be advisable with a focus to implement ethanolic extract of *O.obtusata* as a natural preservative on freshly cut fruits and vegetables.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

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